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Aproximación fenotípica y molecular a la inducción y detección de resistencia a equinocandinas en *Candida glabrata*

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PRESENTADA POR

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APROXIMACIÓN FENOTÍPICA Y MOLECULAR A LA INDUCCIÓN Y DETECCIÓN DE RESISTENCIA A EQUINOCANDINAS EN *Candida glabrata*

Memoria para la obtención del Grado de Doctor, presentado por

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A mi familia

“Interesáos, os lo ruego, en estos lugares sagrados que llamamos laboratorios. Pedid que se les dote suficientemente. Son los templos del porvenir, de la riqueza y del bienestar. Es en ellos donde la humanidad crece, se fortalece y se hace mejor”

Louis Pasteur

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1. *In vitro* exposure to increasing micafungin concentrations easily promotes echinocandin resistance in *Candida glabrata* isolates.
   Bordallo-Cardona MA, Escribano P, Gómez García de la Pedrosa E, Marcos-Zambrano LJ, Cantón R, Bouza E, Guinea J. 2017.
   Antimicrobial Agents and Chemotherapy 61:e01542-16.

2. Low and constant micafungin concentrations may be sufficient to lead to resistance mutations in *FKS2* gene of *Candida glabrata*.
   Bordallo-Cardona MA, Escribano P, Marcos-Zambrano LJ, Díaz-García J, Gómez García de la Pedrosa E, Cantón R, Bouza E, Guinea J. 2018.
   Medical Mycology 56:903-906.

3. Mutant prevention concentration and mutant selection window of micafungin and anidulafungin in clinical *Candida glabrata* isolates.
   Bordallo-Cardona MA, Marcos-Zambrano LJ, Sánchez-Carrillo C, Gómez García de la Pedrosa E, Cantón R, Bouza E, Escribano P, Guinea J. 2018.
   Antimicrobial Agents and Chemotherapy 62:e01982-17.

4. *MSH2* gene point mutations are not antifungal resistance markers in *Candida glabrata*.
   Bordallo-Cardona MA, Agnelli C, Gómez-Núñez A, Sánchez-Carrillo C, Bouza E, Muñoz P, Escribano P, Guinea J. 2018.
   Antimicrobial Agents and Chemotherapy 63:e01876-18.

5. Resistance to echinocandins in *Candida* can be detected by performing the Etest directly on blood culture samples.
   Bordallo-Cardona MA, Marcos-Zambrano LJ, Sanchez-Carrillo C, Bouza E, Munoz P, Escribano P, Guinea J. 2018.
   Antimicrobial Agents and Chemotherapy 62:e00162-18.

6. Detection of echinocandin-resistant *Candida glabrata* in blood cultures spiked with different percentages of *FKS2* mutants.
   Bordallo-Cardona MA, Sánchez-Carrillo C, Bouza E, Muñoz P, Escribano P, Guinea J. 2018.
   Antimicrobial Agents and Chemotherapy 63:e02004-18.

7. Growth kinetics in *Candida* spp.: Differences between species and potential impact on antifungal susceptibility testing as described by the EUCAST.
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1. RESUMEN

Aproximación fenotípica y molecular a la inducción y detección de resistencia a equinocandinas en *Candida glabrata*.

El estudio de la candidemia causada por *C. glabrata* ha recibido gran interés fundamentalmente por el aumento de su incidencia. A esto se le suma el ascenso en la tasa de resistencia a candinas (multirresistencia en algunos casos) en ciertas zonas geográficas, lo que restringe el uso de estos antifúngicos como tratamiento de primera línea. La detección rápida de las cepas resistentes es una necesidad para mejorar el manejo del paciente y su pronóstico.

Esta tesis ha pretendido dar luz a algunos interrogantes sobre el desarrollo y detección de resistencia a equinocandinas en *C. glabrata* mediante estudios estructurados en los siguientes tres capítulos: Capítulo I, Adquisición *in vitro* de resistencia secundaria a equinocandinas en aislados de *C. glabrata* y mecanismos moleculares implicados; Capítulo II, Determinación de parámetros cinéticos de crecimiento *in vitro* en aislados de *Candida* spp.; y Capítulo III, Detección rápida de resistencia a equinocandinas en *Candida* spp. Se ha propuesto la existencia de zonas anatómicas, como el abdomen y las mucosas, que pueden actuar como reservorios ocultos de cepas resistentes, especialmente aislados de *C. glabrata* resistentes a candinas. Esto es debido, principalmente, a la baja penetración de estos antifúngicos en estas localizaciones, conduciendo a una presión del antifúngico sobre las cepas que pueden promover y posteriormente seleccionar aislados mutantes. Paradójicamente, el número de aislados resistentes encontrados en muestras clínicas es bajo. En el Capítulo I se estudió la capacidad de *C. glabrata* de adquirir resistencia tras exposición *in vitro* a micafungina, simulando los espacios anatómicos con bajas concentraciones de candinas, y se determinaron la concentración preventiva de mutantes, la ventana de selección de mutantes y la frecuencia de mutación a equinocandinas en esta especie. Además, se comparó la virulencia entre cepas de *C. glabrata* sensibles y resistentes a equinocandinas mediante el modelo de *Galleria mellonella*. Se ha propuesto que la adquisición de resistencia en *C. glabrata* puede ser debida a defectos en la reparación del ADN, exactamente en el gen *MSH2*. Se estudió la secuencia del gen *MSH2* y se evaluó su relación con el genotipo, con la posible adquisición de resistencia antifúngica *in vitro* e *in vivo* y con el pronóstico del paciente. En resumen, se demostró que *C. glabrata* adquirió con facilidad resistencia a equinocandinas, especialmente en presencia de concentraciones <2 mg/L. Por contra, no se observó un impacto significativo en la virulencia entre cepas de *C. glabrata* sensibles y resistentes a equinocandinas. Por último, se demostró que la presencia de alteraciones en el gen *MSH2* en aislados de *C. glabrata* no se correlacionaba con genotipos específicos, con la adquisición de resistencia antifúngica *in vitro* ni *in vivo*, ni con el uso previo de antifúngicos en los enfermos.

El estudio *in vitro* de los parámetros cinéticos de crecimiento mediante curvas de densidad óptica es otro método para evaluar la patogenicidad. En el Capítulo II se determinaron estos parámetros cinéticos en aislados de *Candida* spp. causantes de

candidemia, para evaluar posibles diferencias inter e intra especie incluyendo, cepas de *C. glabrata* sensibles y resistentes a fluconazol y a equinocandinas. Inclusive, se analizó el crecimiento de especies durante el tiempo de incubación imitando las condiciones del método de EUCAST. Se encontraron diferencias significativas inter e intra especie en los parámetros cinéticos estudiados, siendo *C. glabrata* la especie que mostró la mayor tasa de crecimiento. Además, la resistencia a las equinocandinas en aislados de *C. glabrata* podría tener un impacto en la curva cinética de crecimiento. Al mimetizar la curva con el método de EUCAST, se observó que se pueden anticipar los resultados de sensibilidad en *C. glabrata*, *C. tropicalis* y *C. krusei*.

La detección de resistencia a través de las pruebas de referencia tiene el inconveniente de arrojar resultados no antes de 48-72 horas tras el diagnóstico de la candidemia. En el Capítulo III se evaluó el papel de dos técnicas rápidas de detección de resistencia a las equinocandinas en diferentes especies de *Candida* (el Etest® y las placas de agar que contienen anidulafungina) realizados directamente en hemocultivos positivos. Se concluyó que las placas con anidulafungina son útiles, aunque no de forma rápida, para el cribado de resistencia en *C. glabrata*, y que el Etest® de micafungina y anidulafungina, es un procedimiento rápido y fiable para detectar resistencia en *Candida* spp., incluso en situaciones de coexistencia de cepas de *C. glabrata* sensibles y resistentes a las candinas cuando estas últimas están infrarrepresentadas.

En resumen, esta tesis demostró el potencial de *C. glabrata* para adquirir resistencia a las equinocandinas de una manera fácil y rápida, independiente del gen *MSH2*. Además, el Etest® de equinocandinas realizado directamente de hemocultivos positivos en los laboratorios de Microbiología serviría para anticipar los resultados de sensibilidad.

2. SUMMARY

Phenotypic and molecular approach to the induction and detection of echinocandin resistance in *Candida glabrata*.

The study of *C. glabrata* causing candidemia has notoriously received attention due to the raise in the incidence of the infection. Moreover, an increase in the candin resistance rate (multi-resistance in some cases) has been detected in some geographical areas, thus restraining the use of candins as first-line drugs. Therefore, a rapid detection of resistant isolates should be a must in order to improve the management and prognosis of patients.

This thesis aimed to enlighten some caveats on the acquisition and detection of resistance to echinocandins in *C. glabrata* isolates in studies grouped into the following chapters: Chapter I, *In vitro* resistance secondary acquisition to echinocandins in isolates of *C. glabrata* and molecular mechanisms involved; Chapter II, Determination *in vitro* growth kinetic parameters in *Candida* spp. and Chapter III, Rapid detection of echinocandin resistance in *Candida* spp.

The existence of anatomical sites, such as the abdomen and mucosa, has been suggested as a hidden reservoir of candin-resistant *C. glabrata* isolates. This may be due to the poor penetration of candins that would drive to the insufficient exposure to low concentrations of candins and, consequently, may promote and select resistant isolates later on. Paradoxically, the number of resistant isolates in clinical samples is low. Chapter I focused on the ability of *C. glabrata* isolates to acquire resistance after *in vitro* exposure to micafungin, mimicking anatomical sites where low concentrations of candins are attained, letting assess the mutation frequency, mutant preventive concentration, and mutant selection window to echinocandins in *C. glabrata*. Moreover, virulence between candin-susceptible and -resistant *C. glabrata* isolates was compared by using *Galleria mellonella* model. The acquisition of resistance in *C. glabrata* to multiple antifungal agents may be explained by some defects in DNA repair, exactly in the *MSH2* gene. The sequence of the *MSH2* gene of *C. glabrata* isolates was studied and compared with genotype, possible acquisition of antifungal resistance *in vitro* and *in vivo,* and patient prognosis. In summary, it was shown that the echinocandin resistance acquisition in *C. glabrata* is rapid and easy, especially in the presence of concentrations of candins <2 mg/L. Conversely, no significant impact on virulence between the two groups was observed. Finally, the presence of mutations in *MSH2* gene was not particularly related to specific genotypes, *in vitro*/*in vivo* acquisition of resistance, or previous use of antifungal agents.

The study of *in vitro* growth kinetic parameters after the study of optical density curves poses a method to evaluate the pathogenicity. Chapter II involved studies to characterize the kinetic parameters of a large collection of *Candida* spp. isolates to check on inter and intra species differences, including both susceptible and resistant *C. glabrata* isolates. Growth of all species during the incubation time was analysed mimicking EUCAST method conditions. The study showed statistically significant differences inter- and intra-

species in kinetic parameters, being *C. glabrata* the fastest species in terms of rate growth. Moreover, candin resistance in *C. glabrata* isolates could have an impact on the kinetic growth curve. Replicating the EUCAST conditions in the growth curve, antifungal resistance reports could be speeded up in *C. glabrata*, *C. tropicalis* and *C. krusei* species.

Resistance detection using the gold standard procedures needs 48-72 hours from the diagnosis of candidemia. Chapter III evaluated the role of two rapid echinocandin resistance detection techniques in different species of *Candida* (the Etest® and anidulafungin-containing agar plates) performed directly on positive blood cultures. Anidulafungin-containing plates were useful, but not fast, to screen for resistance in *C. glabrata*; Etest® was a rapid and reliable method for detecting resistance in *Candida* spp., including *C. glabrata*, even when the proportion of mutants was underrepresented.

In summary, this Thesis demonstrated the potential of *C. glabrata* to easily and promptly acquire resistance to echinocandins, regardless the *MSH2* gene. Furthermore, the echinocandin Etest® performed directly from positive blood cultures in Microbiology laboratories would speed susceptibility report up.

3. INTRODUCCIÓN

3.1. La infección fúngica invasiva. Fungemia y candidemia.

La infección fúngica invasiva (IFI) es una infección oportunista, normalmente nosocomial, cuya principal población en riesgo son los pacientes inmunodeprimidos, pacientes críticos ingresados en unidades de cuidados intensivos, pacientes sometidos a cirugía abdominal, o aquellos con neoplasias hematológicas y/o receptores de trasplante (García-Vidal y Carratalà, 2012; Klingspor et al., 2015). Su incidencia ha aumentado significativamente en las últimas décadas y su alta morbi-mortalidad contribuye al aumento del consumo de recursos hospitalarios para su prevención, diagnóstico y tratamiento (Lass-Florl, 2009; Guinea et al., 2014).

La fungemia es la IFI más frecuente y sus agentes etiológicos principales son las levaduras del género *Candida*. La mayoría de las especies de *Candida* son comensales del ser humano, formando parte sobre todo de la microbiota del tracto gastrointestinal, vaginal y de mucosas. La candidiasis invasiva (IFI producida por *Candida* spp.) generalmente ocurre en pacientes con factores de riesgo conocidos (**Tabla 1**) y puede afectar a cualquier localización del organismo, denominándose candidemia si se demuestra la presencia de ésta en el torrente circulatorio o candidiasis invasora profunda si se encuentran afectados órganos profundos (Cervera, 2012). La candidemia es la fungemia más frecuente en pacientes críticos, clasificándose como la cuarta causa más común de infección del torrente sanguíneo. El género *Candida* comprende más de 150 especies, si bien sólo 15 de ellas han sido descritas como causa de candidiasis invasora; de hecho más del 90% de las fungemias están causadas por sólo cinco especies (*C. albicans, C. glabrata, C. parapsilosis, C. tropicalis* y *C. krusei*) (Pfaller et al., 2005; Pappas et al., 2016).

Tabla 1. Factores de riesgo predisponentes para desarrollar candidiasis invasiva. Adaptado de la literatura (Pappas et al., 2018).

Factores de riesgo	
Intrínsecos	**Iatrogénicos**
Diabetes mellitus	Antibióticos, corticoides e inmunosupresores
Pancreatitis	Hemodiálisis o fallo renal
Sepsis	Catéter venoso central
Gravedad de la enfermedad	Nutrición parenteral
Edades extremas	Cirugías (especialmente gastrointestinal)
Colonización por *Candida* spp.	Ventilación mecánica
Perforación gastrointestinal	Estancia prolongada en servicios hospitalarios
Factores de riesgo adicionales en pacientes inmunodeprimidos	
Intrínsecos	**Iatrogénicos**
Enfermedad injerto contra huésped	Transplante de órgano solido
Mucositis y neutropenia profunda	Transplante de células madre

La incidencia de candidemia ha ido aumentando a lo largo del tiempo, tanto en los Estados Unidos de América como en Europa (Guinea et al., 2014; Lockhart et al., 2012; Astvad et al., 2017) y su epidemiología es dependiente de la situación geográfica. En la mayoría de los estudios, *C. albicans* es la especie principal causando entre un 45-70% de los casos (Pfaller et al., 2005; Puig-Asensio et al., 2014b). En el norte de Europa y en los Estados Unidos de América la segunda especie más frecuente es *C. glabrata*, representando el 32% y el 29% de los casos, respectivamente (Lockhart et al., 2012; Astvad et al., 2017). Sin embargo, en países del sur de Europa y Latinoamérica, *C. parapsilosis* es la segunda especie más aislada siendo *C. glabrata* la tercera especie (Guinea et al., 2014; Da Matta et al., 2017), aunque en pacientes con neoplasias hematológicas, *C. glabrata* es el principal patógeno causante de fungemia (Farmakiotis et al., 2014).

Los factores predisponentes para el desarrollo de candidemia por *C. glabrata* no son genuinamente diferentes del resto de especies de *Candida,* pero destacan el uso de antibióticos de amplio espectro (que favorecen la colonización fúngica), la presencia de catéteres y nutrición parenteral, la inmunodepresión, la ruptura de barreras de mucosa, y el uso de quimio y/o radioterapia (Rodrigues et al., 2014). El posible aumento de *C. glabrata* puede explicarse por otros factores más concretos, como el aumento del uso de fluconazol, el envejecimiento de la población y el aumento en el número de pacientes inmunodeprimidos (Rodrigues et al., 2014; Astvad et al., 2017).

El estudio poblacional CANDIPOP, realizado en centros españoles entre 2010 y 2011, mostró datos sobre la epidemiología de la candidemia en España (Guinea et al., 2014; Puig-Asensio et al., 2014b). La distribución de las especies más frecuentes de este estudio se muestra en la **Figura 1**.

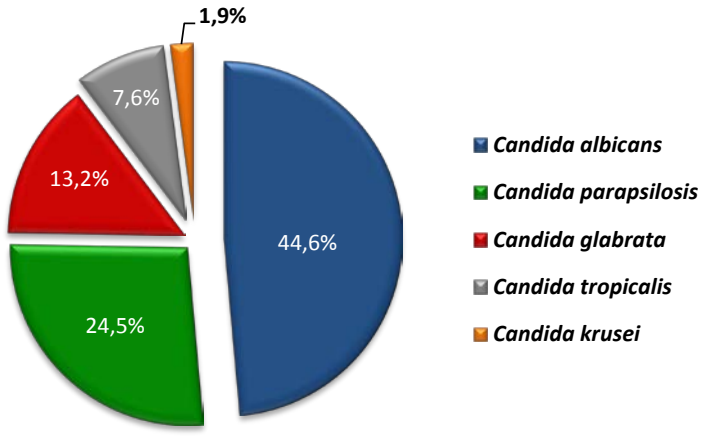


Figura 1. Distribución de las especies más frecuentes causantes de candidemia en España (Estudio CANDIPOP) (Guinea et al., 2014).

La candidemia en España presentó una incidencia de 8,1 casos por cada 100.000 habitantes, siendo especialmente elevada en los pacientes con edades extremas de la vida: <1 año (96,4 casos por cada 100.000 habitantes) y >71 años (26,5 casos por cada 100.000 habitantes). La mortalidad de la candidemia fue elevada (15-35% en adultos y 10-15% en neonatos). La tasa de mortalidad temprana (7 días tras el primer episodio de candidemia) fue del 12,8%, y se asoció a factores relacionados con el manejo del paciente, mientras que la tasa de mortalidad tardía (30 días tras el primer episodio de candidemia) fue mucho mayor (30,6%) y se asoció con factores del huésped y la gravedad de la infección (Puig-Asensio et al., 2014b).

En los últimos años se han publicado estudios que demuestran que la epidemiología de la candidemia está sufriendo cambios, como el aumento de la proporción de casos causados por especies diferentes a *C. albicans*, especialmente por *C. glabrata*. En Dinamarca se ha comunicado una disminución de la proporción de episodios causados por *C. albicans* en 2015 respecto a 2004 (64,4% vs. 42,4%) en paralelo a una duplicación en el número de casos causados por *C. glabrata* (16,5% vs. 34,6%) (Astvad et al., 2017). En España también se ha observado un aumento en el porcentaje de casos causados por *C. glabrata*, sobre todo en pacientes ancianos. Diferentes estudios muestran que en el año 2011 la proporción de casos causados por *C. glabrata* fue del 13,4%, mientras que en estudios anteriores la proporción fue <9% (Pemán et al., 2002; Almirante et al., 2005; Puig-Asensio et al., 2014b). Esta tendencia también ha sido apuntada en los estados de Atlanta y de Baltimore de los Estados Unidos de América durante los años 2008-2011, donde los casos de candidemia causados por *C. albicans* disminuyeron del 52% al 34%. Además, se observó que la proporción de casos causados por *C. glabrata* aumentaron del 12% al 27% (Lockhart et al., 2012) incluso, en unidades de cuidados intensivos estadounidenses (Trick et al., 2002). Igualmente, diversos estudios en Dinamarca, Noruega, Escocia y en los Estados Unidos de América han publicado altas tasas de candidemia causadas por *C. glabrata* (≥20%) (Sandven et al., 2006; Odds et al., 2007; Pfaller et al., 2010; Arendrup et al., 2011a).

Simultáneamente a este cambio epidemiológico de la candidemia, se ha observado un aumento global de la resistencia a candinas y fluconazol en cepas de *C. glabrata* (Alexander et al., 2013; Arendrup y Perlin, 2014). Esto hace necesario conocer la epidemiología local y los patrones de resistencia en cada institución, especialmente a la hora de tomar decisiones terapéuticas correctas. La resistencia a candinas en *C. glabrata* plantea un problema añadido en el manejo de la candidemia, ya que se trata del grupo de antifúngicos de elección (Pappas et al., 2016) en una especie con una intrínseca baja susceptibilidad a azoles; la resistencia a candinas se ha asociado con un peor pronóstico de los pacientes infectados (Alexander et al., 2013; Arendrup y Perlin, 2014).

3.2. Diagnóstico microbiológico de la candidemia.

Es necesario disponer de herramientas diagnósticas precisas, fiables y precoces para iniciar tratamiento antifúngico temprano y mejorar así el pronóstico de los pacientes afectados. Existen métodos diagnósticos microbiológicos basados en el aislamiento del microorganismo o en la detección de biomarcadores en muestras clínicas.

3.2.1. Métodos dependientes del cultivo de *Candida* spp. en muestras de sangre.

El método de referencia para el diagnóstico de la candidiasis invasora se basa en el aislamiento de *Candida* spp. en hemocultivos. Sin embargo, este procedimiento adolece de una baja sensibilidad, ya que un 50% de los casos cursan con hemocultivos negativos (Clancy y Nguyen, 2018). Factores como el volumen de sangre cultivado, la exposición previa a antifúngicos y la especie de *Candida* involucrada, pueden explicar esta baja sensibilidad. Normalmente, la especie de crecimiento más lento en hemocultivos es *C. glabrata* (McCarty y Pappas, 2016), si bien las botellas con medios de cultivo en condiciones anaeróbicas permiten adelantar la detección de este patógeno (Cobos-Trigueros et al., 2013; Gokbolat et al., 2017). Además, es desconocido si los sistemas de hemocultivos permiten la detección de cepas de *C. glabrata* resistentes a antifúngicos en el caso de coexistencia entre cepas salvajes y cepas resistentes, particularmente si estas últimas están infrarrepresentadas. A pesar de estas limitaciones, el hemocultivo sigue siendo una necesidad ya que permite la posterior identificación y el estudio de la susceptibilidad antifúngica de los aislados.

La identificación rápida a nivel de especie de las levaduras causantes de candidemia es importante ya que, el perfil de sensibilidad antifúngica es en gran medida, predecible en base a la especie. La identificación clásica, basada en estudios morfológicos o en las características bioquímicas de las levaduras, son métodos muy utilizados en la rutina del laboratorio de Microbiología, aunque desafortunadamente la identificación clásica se demora 48-72 horas tras el cultivo de la muestra. Los medios cromogénicos han sido diseñados para la identificación de las especies del género *Candida* más frecuentes que producen candidiasis invasora. El medio CHROMagar *Candida*® es el medio que mejor diferencia las cinco especies más frecuentes, manifestándose *C. glabrata* con un color rosa-violeta. Hay otros métodos comercializados que utilizan la asimilación de los nutrientes por parte de los aislados, entre los que destacan AuxaColor, API 20C AUX®, Sistema Vitek2® y Rapid Yeast Identification Panel MicroScan® (Neppelenbroek et al., 2014; Alam et al., 2014). Además, para la identificación exclusiva de *C. glabrata,* existe un sistema enzimático comercializado denominado GLABRATA RTT (Fumouze Diagnostics, Levallois Perret, Francia) (Freydiere et al., 2003).

La identificación de levaduras basadas en técnicas de biología molecular se apoya en la reacción en cadena de la polimerasa (PCR), siendo más precisa que la identificación

morfológica convencional ya que, permite la identificación de especies crípticas filogenéticamente relacionadas (Kurtzman et al., 2015). Los genes ribosomales que codifican las subunidades 5,8S-18S-26S están dispuestos en tándem formando unidades de transcripción que se repiten entre 100 y 200 veces en el genoma de las levaduras. En cada unidad de transcripción existen espaciadores internos denominados ITS (**I**nternal **T**ranscribed **S**pacer), que no se transcriben y varían lo suficiente como para poder distinguir entre diversas especies de *Candida*. Para la identificación inequívoca de levaduras se amplifican las regiones ITS 1 (gen 18S y 5,8S) y la región ITS 2 (gen 5,8S y 26S) con cebadores universales (**Figura 2**).

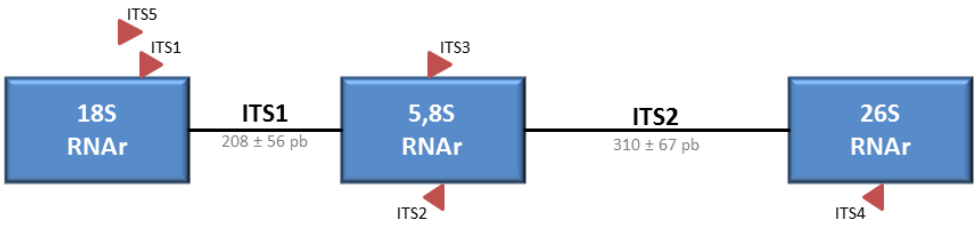


Figura 2. Regiones ITS ("Internal Transcribed Spacer") de las levaduras con sus cebadores correspondientes.

Otra técnica desarrollada es el FilmArray®, consistente en un panel que mediante múltiples PCRs permite la identificación directamente sobre el hemocultivo positivo de 24 microorganismos (incluidas las cinco especies más frecuentes de *Candida*) en aproximadamente 1 hora. Ha demostrado una alta sensibilidad y especificidad (>99%) en la identificación de *Candida* (Salimnia et al., 2016) y la posibilidad de detectar infecciones polimicrobianas (Ibáñez-Martínez et al., 2017).

Los métodos proteómicos, cada vez más disponibles en los hospitales españoles, permiten la identificación de levaduras de una manera más rápida ya que se puede realizar además, sobre el hemocultivo positivo. La espectrometría de masas MALDI-TOF MS (**m**atrix-**a**ssisted **l**aser **d**esorption **i**onization-**t**ime **o**f **f**light **m**ass **s**pectrometry), genera un espectro característico de especie mediante el análisis de las proteínas ribosomales de las levaduras. Este espectro se compara con una base de datos pre-establecida, indicando la especie en cuestión mediante una puntuación determinada. Potencialmente, este sistema puede emplearse para realizar pruebas de sensibilidad antifúngica (Croxatto et al., 2012; Posteraro y Sanguinetti, 2014).

3.2.2. Métodos de diagnóstico independientes del cultivo.

Estos métodos han sido desarrollados fundamentalmente para paliar las limitaciones de los hemocultivos. La primera prueba diagnóstica independiente del cultivo, fue la detección en suero de antígenos o anticuerpos del género *Candida* (Clancy y Nguyen, 2013). Sin embargo, la búsqueda de antígenos está muy limitada por la baja concentración sérica y rápido aclaramiento desde el torrente sanguíneo (Ellepola y Morrison, 2005). Los métodos más conocidos son la detección de los componentes de la pared celular de las levaduras, como el antígeno de manano y anticuerpos anti-manano (Platelia™ *Candida*), la detección del 1,3-β-D-glucano, y los anticuerpos antimicelio CAGTA (***C**andida **a**lbicans* **g**erm **t**ube **a**ntibody) (Mikulska et al., 2010; Bassetti et al., 2016; McCarty y Pappas, 2016; Clancy y Nguyen, 2018). Otros métodos menos implementados son el PNA-FISH (**P**eptide **N**ucleic **A**cid **F**luorescent **I**n **S**itu **H**ybridation), técnica de hibridación de fluorescencia *in situ* mediante sondas de ácidos nucleicos peptídicos para la identificación rápida de las cinco especies de *Candida* spp. más frecuentes desde el hemocultivo positivo (Gorton et al., 2014), y el T2*Candida*®. Este último detecta las cinco especies más comunes de *Candida* directamente en sangre completa, mediante PCR y posterior análisis mediante resonancia magnética, disminuyendo los tiempos de diagnóstico (Clancy y Nguyen, 2018). En la **Figura 3** se muestra el tiempo requerido para alcanzar resultados por los diferentes métodos de identificación.

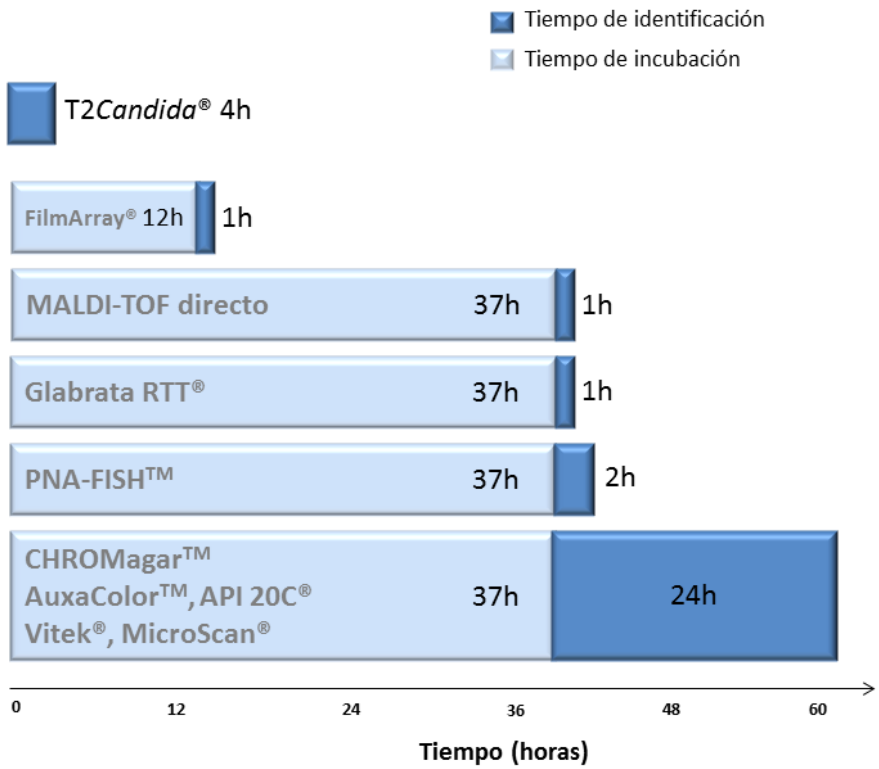


Figura 3. Comparación de los métodos de identificación según el tiempo requerido para la obtención de resultados. Adaptado de la literatura (Ibánez-Martínez et al., 2017).

3.3. Manejo y tratamiento antifúngico de la candidemia.

Para ayudar en el manejo de la candidiasis se han publicado dos guías, una de la Sociedad Americana de Enfermedades Infecciosas (IDSA) y otra de la Sociedad Europea (ESCMID) (Cornely et al., 2012; Pappas et al., 2016) con recomendaciones específicas sobre la prevención y una rápida y adecuada iniciación de la terapia antifúngica (Pappas et al., 2018). Los agentes antifúngicos que se utilizan para el tratamiento de la candidemia son las candinas (micafungina, anidulafungina y caspofungina), los azoles (fluconazol y voriconazol) y la anfotericina B (Cornely et al., 2012; Pappas et al., 2016). La selección del fármaco inicial se debe basar en la exposición previa o en la intolerancia del paciente al antifúngico, la gravedad de la enfermedad y en la susceptibilidad del agente etiológico causante de la infección (Pappas et al., 2018). Las limitaciones de los métodos diagnósticos conducen a un elevado número de tratamientos empíricos, y en paralelo, a un uso innecesario de antimicóticos en pacientes sin candidiasis invasiva (Pappas et al., 2018).

En el caso de que la candidemia esté relacionada con el catéter, la retirada del catéter central es recomendable, ya que mejora el pronóstico del paciente. Sin embargo, esta decisión debe ser individualizada después de valorar el beneficio/riesgo y de la posibilidad de que éste sea o no el foco de infección (Puig-Asensio et al., 2014a). Las guías ESCMID recomiendan que si no se puede retirar el catéter se use una candina o la anfotericina B, debido a su actividad anti-biopelícula (Cornely et al., 2012) ya que hasta el momento, la técnica de sellado no está recogida en las guías para el tratamiento de infección del catéter por el género *Candida*.

En la **Figura 4** se muestra el lugar de acción de los diferentes grupos de antifúngicos mencionados anteriormente.

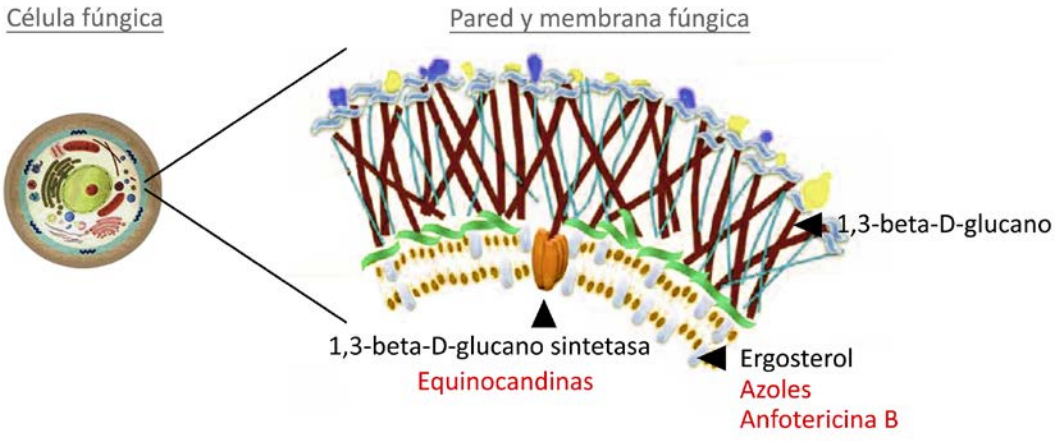


Figura 4. Lugar de acción de los antifúngicos más empleados en la clínica. Adaptado de la literatura (Diamond, 2001; Maertens J, 2002).

3.3.1. Candinas.

Las candinas son lipopéptidos que muestran una actividad fungicida frente a las diferentes especies de *Candida*. Interrumpen la formación del 1,3-β-D-glucano de la pared celular mediante la inhibición de manera no competitiva de la enzima 1,3-β-D-glucano-sintetasa (específicamente inhibiendo la subunidad catalítica Fks1p, codificada por los tres genes *FKS1, FKS2* y *FKS3*) provocando una alteración osmótica y por consiguiente una lisis celular (Denning, 2003; Chen et al., 2011; Bassetti et al., 2016). Tienen un amplio espectro de acción, actuando incluso sobre las biopelículas. Su perfil de tolerabilidad y seguridad es alto, ya que el 1,3-β-D-glucano no forma parte de las células humanas, las interacciones con otros fármacos son limitadas y la toxicidad renal y hepática es baja (Kofla y Ruhnke, 2011). Su formulación es intravenosa ya que, debido a su alto peso molecular, su absorción oral es pobre (Chen et al., 2011). Se recomiendan como terapia de primera línea para el tratamiento de candidiasis invasiva, sobre todo en pacientes que han sido expuestos anteriormente a azoles o colonizados por especies de *Candida* con susceptibilidad reducida a los azoles, como es el caso de *C. glabrata* (Pfaller et al., 2003; Pappas et al., 2016). Existen pequeñas diferencias farmacocinéticas entre las tres candinas, aunque no se han encontrado diferencias en cuanto a su eficacia clínica, por lo que las tres son intercambiables (Eschenauer et al., 2007; Pappas et al., 2007; Pappas et al., 2016). Su desventaja es que no alcanzan concentraciones terapéuticas efectivas en ciertos tejidos como, ojos, sistema nervioso central, orina, endocardio y peritoneo (Bassetti et al., 2016).

3.3.2. Azoles.

Los azoles son un grupo de antifúngicos fungistáticos que actúan como inhibidores de la enzima 14-α-lanosterol desmetilasa (codificada por el gen *ERG11*) del grupo de las enzimas del citocromo P450. Esta enzima es clave para la formación del ergosterol, un componente mayoritario de la membrana de las células fúngicas, cuya modificación altera la fluidez de la membrana. Presentan una amplia actividad contra la mayoría de *Candida* spp., pueden administrarse de forma oral o intravenosa y son generalmente bien tolerados (Odds et al., 2003). Uno de los azoles más utilizados es el fluconazol, fármaco de elección para el tratamiento de la candidemia, hasta que alrededor del año 2003 se incorporaron las candinas, y pasó a ser una terapia de segunda línea. Sin embargo, se sigue prefiriendo en casos específicos, como en la endolftalmitis, meningitis y candidiasis del tracto urinario, donde las candinas no alcanzan concentraciones suficientes (Pappas et al., 2018). El voriconazol puede ser una alternativa, sobretodo en pacientes infectados con aislados que tengan una sensibilidad disminuida al fluconazol, como es el caso de *C. glabrata* (Pappas et al., 2018), aunque existen casos de resistencia cruzada con otros azoles (Pfaller et al., 2012b) y su principal desventaja es que debe ser monitorizado (Troke et al., 2011).

Además, existen dos fármacos más novedosos, el posaconazol, que exhibe un espectro más amplio y una actividad más potente comparándolo con el fluconazol (Pfaller et al., 2004; Pfaller et al., 2013a) y el isavuconazol, que tiene una excelente actividad *in vitro* contra *C. glabrata*, aunque existen pocos datos clínicos que apoyen su uso habitual de pacientes con candidemia (Pappas et al., 2018).

3.3.3. Anfotericina B

La anfotericina B es un fármaco fungicida de la familia de los polienos, que se fija a los esteroles de la membrana de las células eucariotas, teniendo más afinidad por el ergosterol de los hongos que por el colesterol de las células humanas. Provoca alteraciones en la fisiología de la membrana mediante la formación de poros, originando una despolarización de la membrana y un aumento en la permeabilidad, lo que lleva a la muerte celular (Moen et al., 2009). Presenta un amplio espectro contra hongos patógenos y se administra de forma intravenosa por su alto grado de hidrofobia y baja absorción a nivel gastrointestinal (Odds et al., 2003). Actualmente, existen formulaciones lipídicas que mejoran la tolerancia a nivel de la infusión del medicamento y disminuyen la toxicidad renal, ya que tienen mejorado su perfil de solubilidad, permitiendo dosis diarias y totales más elevadas en un menor tiempo (Moen et al., 2009).

Hoy en día, las guías de la IDSA recomiendan para el tratamiento de la candidemia, independientemente de la especie, una terapia inicial con una candina (Pappas et al., 2016). El fluconazol se podría considerar en pacientes hemodinámicamente estables, sin exposición previa a azoles e infectados con especies sensibles a este antifúngico. La duración del tratamiento está determinada por la respuesta clínica y microbiológica a la terapia, y en ausencia de afectación orgánica debe ser de 14 días tras el primer hemocultivo negativo, junto a la resolución de los signos de infección en el paciente (Pappas et al., 2016). Específicamente, para el tratamiento de *C. glabrata*, una especie con sensibilidad reducida al fluconazol, se recomienda como tratamiento inicial una candina y como alternativa la anfotericina B, y para desescalar a un antifúngico oral, la terapia más óptima es voriconazol o fluconazol a altas dosis (Pappas et al., 2016).

3.3.4. Nuevos antifúngicos para el tratamiento de la candidemia.

A pesar de las opciones terapéuticas comentadas, siguen existiendo limitaciones como la toxicidad de los azoles, las interacciones con otros medicamentos, la vía de administración intravenosa de las candinas y la falta de actividad frente a especies resistentes. Todo ello justifica la necesidad de desarrollar nuevas moléculas. Las moléculas más prometedoras son la rezafungina (CD101) y el SCY-078, ya que al ser inhibidores de la síntesis de glucano, mejorarían el tratamiento de primera línea.

La rezafungina es una nueva equinocandina (análogo de la anidulafungina) cuya administración semanal (gracias a sus propiedades farmacocinéticas y farmacodinámicas) disminuiría la hospitalización de los pacientes, y con ello el coste hospitalario. Además, aunque el espectro de acción y la tasa de mutación sea similar a las otras candinas, podría ayudar a resolver los problemas de resistencia en *Candida* spp. por las altas concentraciones tisulares alcanzadas (Pfaller et al., 2016; Bader et al., 2018). En modelos murinos, ha demostrado una excelente penetración abdominal, manteniéndose sostenible a una alta concentración en la fase inicial de la infección, por lo que podría llegar a penetrar mejor en tejidos lesionados que las candinas tradicionales (Zhao et al., 2017). El SCY-078 es un inhibidor de la 1,3-β-D-glucano-sintetasa, con una estructura química diferente a las candinas y posee actividad contra *Candida* spp. resistentes a estos antifúngicos (Jiménez-Ortigosa et al., 2014). Su gran ventaja es que su administración puede ser oral además de la intravenosa, lo que podría facilitar el manejo y la adherencia de los pacientes ambulatorios. Aunque no es un fármaco extremadamente activo frente a *C. glabrata*, podría tener un impacto clínico en áreas donde la tasa de *C. glabrata* resistentes a las candinas está en aumento (Wiederhold et al., 2018). Además, parece ser prometedor en el tratamiento de las biopelículas (Marcos-Zambrano et al., 2017b).

El VT-1161 es un azol que, a diferencia de los anteriores, se une con mayor afinidad al citocromo fúngico que al humano, siendo activo frente aislados resistentes a fluconazol o a candinas (González-Lara et al., 2017). El APX001A (previamente denominado E1210) es la molécula activa del APX001, que inhibe la enzima inositol aciltransferasa de los hongos evitando la maduración proteica y el crecimiento fúngico. Tiene un amplio espectro, incluyendo *Candida* spp. resistentes a fluconazol y candinas y su administración podría ser oral o intravenosa (Pfaller et al., 2011a). *In vitro* e *in vivo,* es igualmente o más eficaz que anfotericina B, anidulafungina, micafungina, fluconazol y voriconazol, por lo que es un agente útil en infecciones causadas por *C. glabrata* (Arendrup et al., 2018; Zhao et al., 2018). No parece tener resistencias cruzadas y penetra en sitios anatómicos de difícil acceso para otros antifúngicos, como en el sistema nervioso central, pulmón y riñón (Hager et al., 2018).

3.4. Estudio de la sensibilidad antifúngica.

El estudio de la sensibilidad de los hongos a los antifúngicos es una de las funciones más importantes de los laboratorios de micología. Se aconseja realizar las técnicas de susceptibilidad antifúngica sobre cepas clínicamente significativas, especialmente si son recurrentes debido al fracaso terapéutico. Además, es importante para la vigilancia epidemiológica y de la resistencia.

El principal objetivo de estas técnicas es evaluar la respuesta *in vitro* de un hongo a varios antifúngicos, para detectar así la presencia de cepas resistentes e instaurar el mejor tratamiento posible (Arendrup MC et al., 2017). Los comités europeo (EUCAST - **Eu**ropean **C**ommittee on **A**ntimicrobial **S**usceptibility **T**esting) y americano (CLSI - **C**linical **L**aboratory **S**tandards **I**nstitute) han publicado procedimientos de referencia estandarizados y reproducibles para la determinación de la sensibilidad antifúngica (Arendrup MC et al., 2017; Clinical and Laboratory Standards Institute, 2017).

3.4.1. Métodos basados en la microdilución en caldo.

Las técnicas de microdilución en caldo son las de referencia para determinar la susceptibilidad antifúngica *in vitro* a las levaduras. Se utilizan para establecer la **c**oncentración **m**ínima **i**nhibitoria (CMI) de los antifúngicos utilizados habitualmente en la clínica, o bien para estudiar nuevos agentes antimicóticos. Estas técnicas consisten en evaluar la capacidad de crecimiento del hongo en las placas de microdilución, donde se inoculan diluciones seriadas del antifúngico junto a suspensiones conocidas de la levadura a estudio, comparándolo con un pocillo control sin antifúngico.

La CMI es la concentración más baja de un agente antifúngico capaz de inhibir el crecimiento de un hongo cuando se compara con el control libre de antifúngico, expresándose en mg/L. Se define la CMI como un 50% o superior de inhibición en el caso de los azoles y las candinas y una inhibición total (100%) en el caso de la anfotericina B. Los comités CLSI y EUCAST han desarrollado un procedimiento de microdilución muy similar. Sin embargo, presentan alguna diferencia metodológica que hace que el método proporcionado por EUCAST sea más objetivo y fácil de interpretar ya que, la lectura de la CMI se realiza mediante un espectrofotómetro (Arendrup MC et al., 2017; Clinical and Laboratory Standards Institute, 2017) (**Figura 5**).

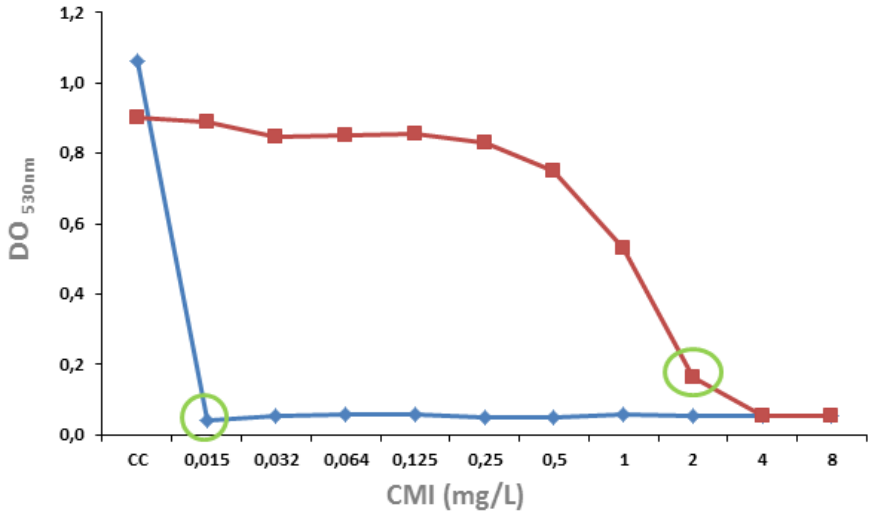


Figura 5. Ejemplo de la lectura espectrofotométrica de la CMI mediante el método EUCAST. Curvas de inhibición del crecimiento de *C. glabrata* indicando sensibilidad (curva azul, CMI= 0,015 mg/L) y resistencia (curva roja, CMI= 2 mg/L) a micafungina. DO, densidad óptica; CC, control de crecimiento (DO del pocillo sin antifúngico).

Un método comercial de microdilución en caldo muy utilizado en los laboratorios es el Sensititre YeastOne® (TREK Diagnostic Systems, United Kingdom). Su metodología es muy similar a la del CLSI, sin embargo un mismo panel multipocillo lleva muchos antifúngicos con un sustrato cromogénico, facilitando la interpretación de la CMI.

La CMI informa sobre probabilidad de éxito terapéutico de un paciente tratado con el agente antifúngico en cuestión (Arendrup MC et al., 2017). El análisis poblacional de las CMIs permite clasificar a las cepas en clínicamente sensibles, intermedias, o resistentes de acuerdo a los puntos de corte clínicos establecidos. Las cepas clasificadas como sensibles se asocian con una alta probabilidad de éxito terapéutico, las clasificadas como resistentes se asocian con una alta probabilidad de fracaso terapéutico y las intermedias (sensible dosis dependiente) requiere una exposición mayor al fármaco para asegurar respuesta clínica. Los puntos de corte clínicos son específicos de cada especie y están basados en un conjunto de datos epidemiológicos, farmacodinámicos y clínicos (Cuenca-Estrella et al., 2013).

El paso previo para el establecimiento de los puntos de corte clínicos son el desarrollo de los puntos de corte epidemiológicos o ECOFF, que se definen como el valor de CMI que limita a la población salvaje (aislados sin mecanismos de resistencia adquirida). CMIs por debajo del ECOFF agrupan al 95% de las cepas de una especie (Arendrup et al., 2014). Los puntos de corte clínicos son parámetros de uso clínico capaces de ayudar en la toma de decisiones terapéuticas, mientras que los ECOFF, sirven como medida más sensible para la detección de aislados con sensibilidad reducida o con mecanismos de resistencia a un agente antifúngico (Turnidge y Paterson, 2007).

En la **Figura 6** se muestra un ejemplo de distribución de CMIs indicando los puntos de corte clínicos y el punto epidemiológico, en este caso es para fluconazol y *C. glabrata*.

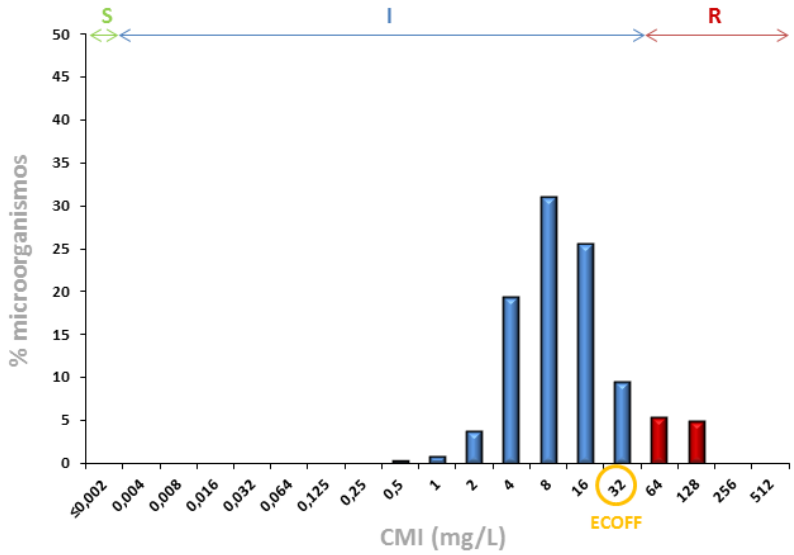


Figura 6. Distribución de CMIs para *C. glabrata* frente a fluconazol. Puntos de corte clínicos, S ≤0,002 mg/L; I =0,004-32 mg/L; R>32 mg/L. El punto de corte epidemiológico ECOFF= 32 mg/L, corresponde a la CMI que inhibe ≥95% de las cepas estudiadas y cepas con una CMI ≤32 mg/L son de tipo salvaje. Adaptado de http://www.eucast.org (898 cepas de 12 sitios anatómicos); Enero 2019.

CLSI y EUCAST han propuesto puntos de corte para las especies de *Candida* más frecuentes y los antifúngicos recomendados. Sin embargo, EUCAST ha encontrado variaciones significativas en las CMIs de caspofungina entre los laboratorios, por lo que todavía no ha establecido puntos de corte para este antifúngico, debido al riesgo de clasificar erróneamente a los aislados (Espinel-Ingroff et al., 2013; European Committee on Antimicrobial Susceptibility Testing, 2018). Por otro lado, CLSI muestra que los puntos de corte para caspofungina pueden aumentar la tasa de los aislados no sensibles, especialmente en *C. glabrata* (Eschenauer et al., 2014). Estos hallazgos llevan a que los dos comités propongan utilizar la micafungina y la anidulafungina como marcadores de resistencia a candinas (Arendrup et al., 2012; Pfaller et al., 2014a; Pfaller et al., 2014b). En la **Tabla 2** se muestran los puntos de corte clínicos de *C. glabrata* a los antifúngicos empleados tanto en terapia inicial como en terapia para desescalar, según la metodología de EUCAST.

Tabla 2. Puntos de corte clínicos (mg/L) para *C. glabrata* a los antifúngicos recomendados según la metodología de EUCAST (European Committee on Antimicrobial Susceptibility Testing, 2018). Enero del 2019.

	Categoría clínica*	
	S≤	R>
Micafungina	0,032	0,032
Anidulafungina	0,064	0,064
Anfotericina B	1	1
Fluconazol	0,002	32

*La categoría intermedia comprende los valores de CMI entre las categorías sensible y resistente.

3.4.2. Métodos de difusión.

Son métodos *in vitro*, de realización más simplificada y adaptados a la actividad del laboratorio de microbiología. Se fundamentan en la preparación de un inóculo estandarizado de la cepa a estudiar, que se siembra sobre una placa de agar, sobre la que se deposita un soporte con antifúngico desecado que se solubiliza una vez en contacto con el medio acuoso y produce una inhibición cuantificable del crecimiento del hongo. El método de difusión en disco es una técnica estandarizada por el CLSI para especies de *Candida,* que consiste en la medida del halo de inhibición del crecimiento de la levadura sembrada en la placa de agar producido alrededor del disco impregnado de antifúngico (Clinical and Laboratory Standards Institute, 2009); este método aporta resultados cualitativos pero no permite la obtención de la CMI directamente. Otro método de difusión para levaduras, muy utilizado en los laboratorios de microbiología por su comodidad es el Etest®, que mantiene el mismo principio que el anterior pero, en vez de un disco consiste en una tira plástica impregnada con un gradiente de concentraciones de antifúngico cuya difusión en la placa de agar genera una elipse de inhibición que permite la obtención de la CMI así como su posterior interpretación (**Figura 7**).

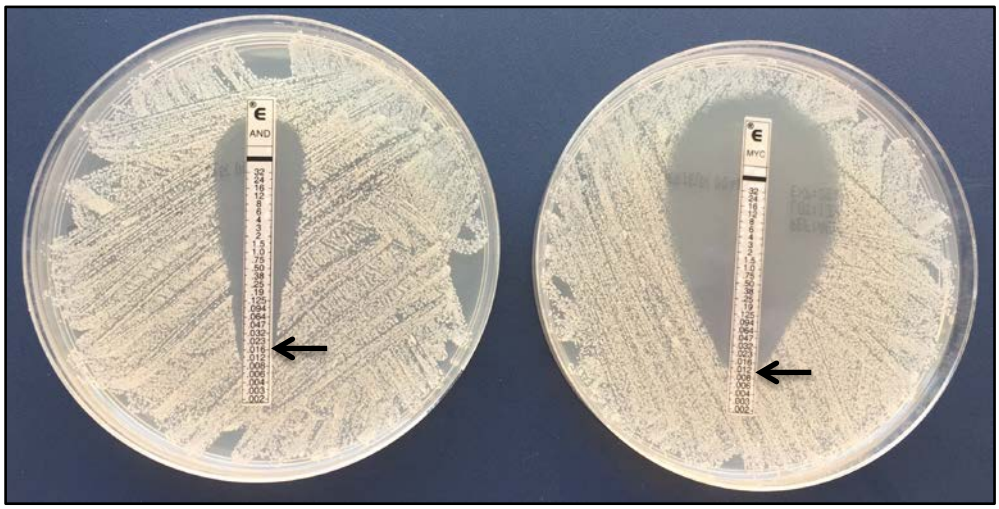


Figura 7. Ejemplo de la obtención de CMIs mediante la utilización del Etest®.

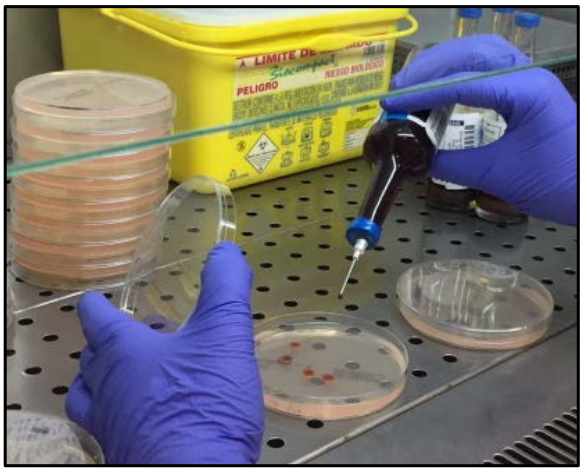
3.4.3. Métodos rápidos de estudio de la sensibilidad antifúngica.

Dado que la principal función de cualquier método para el estudio de la sensibilidad a los antifúngicos es la detección de cepas resistentes y/o no salvajes, dicha detección debe hacerse en el menor tiempo posible desde el diagnóstico. Los métodos anteriormente comentados están estandarizados para estudiar la sensibilidad de las cepas en cultivo puro, lo que inevitablemente conlleva un retraso en la obtención de resultados de 48 y 72 horas desde el diagnóstico de la candidemia. Se han estudiado potenciales modificaciones de los métodos de sensibilidad para anticipar los resultados utilizando el Etest®, las placas de agar con antifúngicos, el MALDI-TOF MS y la detección molecular directamente sobre muestra clínica.

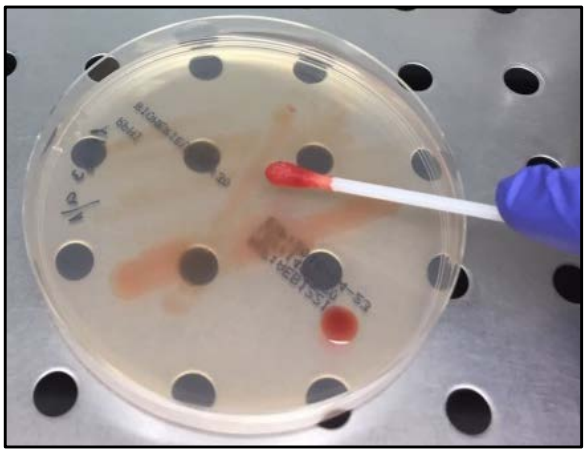
Teóricamente, el Etest® es un método independiente del inóculo, por lo que su realización directamente sobre muestra clínica es posible. En estudios previos, el grupo demostró que la realización del Etest® directamente sobre muestras de hemocultivos positivos con levaduras, permitía la obtención de la CMI a los azoles tras 24 horas del diagnóstico de la candidemia (Guinea et al., 2010; Escribano et al., 2017).

La realización de este método es muy simple, tal y como se muestra en la **Figura 8**. El primer paso es inocular la sangre del paciente con sospecha de candidemia en las botellas destinadas para su cultivo y esperar a que los hemocultivos se vuelvan positivos **(Figura 8.1)**; el segundo paso es dejar caer unas gotas del hemocultivo positivo sobre la placa de RPMI 1640 **(Figura 8.2)**; el tercer paso, extender con una torunda las gotas del hemocultivo por toda la placa de RPMI 1640 **(Figura 8.3)**; el cuarto paso, colocar correctamente la tira del Etest® del antifúngico elegido e incubar a 35 °C durante 24 horas **(Figura 8.4)**; y por último, se procederá a la lectura del halo de inhibición para la obtención de la CMI del aislado **(Figura 8.5)**.

8.1



8.2



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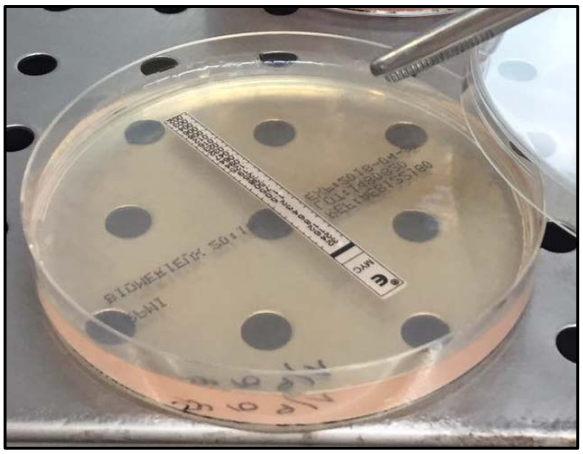

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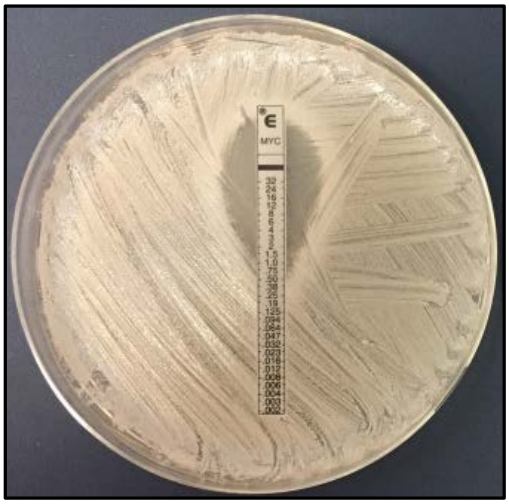

8.5

Figura 8. Pasos para realizar el Etest® directamente sobre hemocultivos positivos en levaduras.

La detección de resistencia a azoles en cepas de *C. glabrata* a través de este método es comparable a los resultados obtenidos por los métodos de referencia del CLSI y del EUCAST (Guinea et al., 2010; Escribano et al., 2017). Sin embargo, dado que las candinas son los antifúngicos de elección para el tratamiento de la candidemia, sería de utilidad saber si este procedimiento sería útil para la detección de resistencia a candinas en *Candida* spp.

El uso de placas de agar con antifúngico ha demostrado ser útil para la detección de cepas de *Aspergillus fumigatus* resistentes a azoles, mostrando un elevado acuerdo con los métodos de referencia y siendo un método de fácil implementación en los laboratorios de rutina (Arendrup et al., 2017; Guinea et al., 2018). Sin embargo, no hay estudios que evalúen este método en *C. glabrata* para detectar la presencia de cepas resistentes a candinas.

El MALDI-TOF MS ha sido utilizado principalmente para la identificación de aislados, aunque presenta un elevado potencial para otras aproximaciones, como la detección de resistencia antifúngica a azoles y candinas, si bien esto último requiere aún mayor estandarización. Este procedimiento ha sido utilizado sobre botellas de hemocultivos positivos o bien sobre cultivos puros (Sanguinetti y Posteraro, 2017; Croxatto et al., 2012; Posteraro y Sanguinetti, 2014).

Se han desarrollado PCRs que utilizan sondas de oligonucleótidos específicas para cada mutación en los genes *FKS* de cepas en cultivo puro, pero técnicamente este procedimiento es difícil de estandarizar (Sanguinetti y Posteraro, 2017). Por el contrario, las técnicas de PCR multiplex parecen ser herramientas más eficaces a la hora de detectar la resistencia a las candinas (Pham et al., 2014a; Zhao et al., 2016). Diversos autores, informaron de una concordancia de un 100% en la detección de mutaciones en los genes *FKS1* y *FKS2* en cerca de 200 aislados de *C. glabrata* (Zhao et al., 2016). Sin embargo, estas técnicas no se han podido validar aún para la detección de resistencia a azoles, debido a la complejidad de los mecanismos de resistencia y todavía no están disponibles de forma rutinaria en los laboratorios de microbiología (Pfaller, 2012). Teóricamente, la aplicación de técnicas moleculares directamente en muestras de sangre ofrece la posibilidad de detectar la presencia de determinados genes de resistencia a los antifúngicos, facilitando la elección del tratamiento más adecuado. La **Figura 9** muestra los tiempos de respuesta de los métodos aquí comentados.

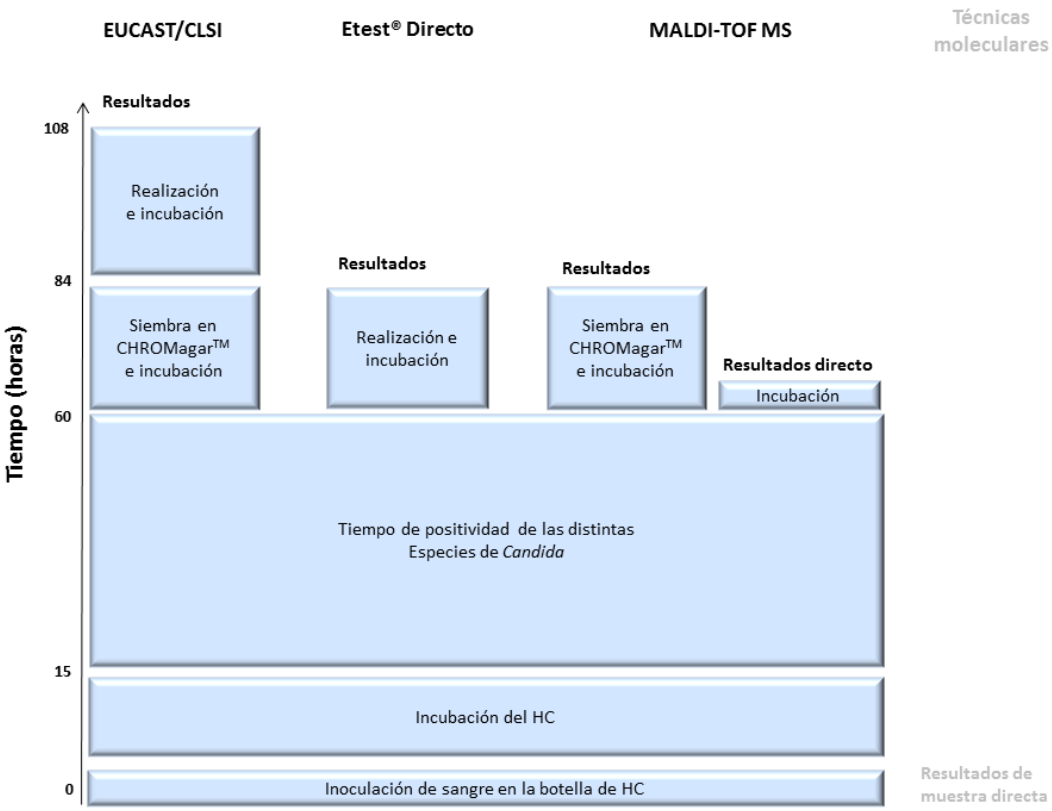

Figura 9. Tiempos de respuesta de los diferentes métodos para el estudio de la sensibilidad a los antifúngicos en *Candida* spp. En gris, tiempo de respuesta de futuras aplicaciones.

3.4.4. Resistencia antifúngica en *C. glabrata*.

El desarrollo en la última década de antifúngicos menos tóxicos, incluyendo las candinas, ha promovido su utilización en profilaxis, tratamiento empírico y terapia dirigida, lo que ha llevado a un aumento de resistencia, así como a una alteración de la epidemiología, limitando las opciones terapéuticas y provocando un efecto devastador en el paciente infectado, siendo *C. glabrata* una de las especies más afectadas (Lortholary et al., 2011; Alexander et al., 2013; Arendrup y Perlin, 2014; Fekkar et al., 2014).

El desarrollo de resistencia antifúngica es un proceso complejo, que implica al huésped, a los antifúngicos y a los factores microbianos, teniendo como consecuencia el fallo terapéutico. Los pacientes más propensos a una falta de respuesta en el tratamiento son los inmunodeprimidos, ya que su respuesta inmune ante la infección es muy débil (Brown y Netea, 2012). Otros factores involucrados son el uso de catéteres permanentes, válvulas cardiacas artificiales y otros dispositivos implantados, que promueven la colonización del material en forma de biopelículas, siendo éstas menos accesibles a la penetración de los antifúngicos (Larkin et al., 2018). Además, los antifúngicos no llegan a alcanzar concentraciones terapéuticas adecuadas en algunas zonas del organismo, como el

abdomen y/o las mucosas, pudiendo actuar como reservorios ocultos de resistencia, sobre todo de *C. glabrata* resistentes a candinas (Shields et al., 2014; Jensen et al., 2015; Grau et al., 2015). Esto puede ser debido a que, en algunos casos, los fármacos que están altamente unidos a proteínas séricas, como las candinas, se pueden comportar como fungicidas *in vitro* y como fungistáticos *in vivo* (Kovacs et al., 2014). Uno de los factores principales que impulsan la aparición de resistencia, es la administración prolongada del antifúngico y el uso de antifúngicos como profilaxis (Thompson et al., 2008; Alexander et al., 2013; Beyda et al., 2014). Sin embargo, para *C. glabrata* está descrito que cortos periodos de tiempo, tras el comienzo con el tratamiento, son suficientes para promover la aparición de resistencia a las candinas y posteriormente, estas cepas resistentes, puedan transferirse entre pacientes hospitalizados (Lewis et al., 2013; Alexander et al., 2013).

Los mecanismos de resistencia microbiológica a un antifúngico pueden ser de dos tipos: primarios (intrínseca de una especie) o secundarios (adquiridos por cepas de una especie fundamentalmente sensible al antifúngico en cuestión). La resistencia primaria o intrínseca es una resistencia no adquirida, no relacionada con la exposición previa al antifúngico y es inherente a todos los representantes de una misma especie, favoreciendo el fallo clínico. Algunas de las especies de *Candida*, como *C. glabrata*, presentan una inherente falta de susceptibilidad a azoles (Pfaller y Diekema, 2004; Perlin et al., 2015). El tratamiento con fluconazol de infecciones invasoras por *C. glabrata* no se recomienda de inicio, y se reserva para desescalar usándolo siempre en dosis altas y cuando la CMI de la cepa sea <32 mg/L (Cornely et al., 2012; Pappas et al., 2016). La resistencia secundaria o adquirida se refiere a la adquisición del mecanismo de resistencia durante la exposición al tratamiento, aunque también se ha descrito que puede ocurrir en pacientes que no han recibido tratamiento antifúngico, debido a la transmisión paciente-paciente de microorganismos resistentes (Pinhati et al., 2016). Aunque la resistencia a antifúngicos es numéricamente baja, lo más habitual es que las cepas sean intrínsecamente resistentes (Pfaller et al., 2011c). Como se ha comentado, sin embargo, se está comunicando un aumento de resistencia a candinas (mecanismo secundario) en *C. glabrata* implicando fallo terapéutico y además, resistencia a los azoles (Alexander et al., 2013; Pham et al., 2014b; Beyda et al., 2014; Arendrup y Perlin, 2014; Cleveland et al., 2015).

Normalmente, los mecanismos de resistencia implican una menor penetración del fármaco (o bien por baja permeabilidad o por extrusión a través de bombas de eflujo) (Perlin et al., 2015) o bien modificaciones en la diana de acción. Las bases moleculares de la resistencia son específicas para cada clase de antifúngicos.

3.4.4.1. Resistencia a azoles en *C. glabrata*.

El uso generalizado de los azoles, tanto en profilaxis como en tratamiento, ha conducido a un aumento de la resistencia a estos antifúngicos (Perlin, 2015b), siendo muy

notorio en especies diferentes de *C. albicans* (Ostrosky-Zeichner et al., 2017; Wiederhold, 2017). Esto es preocupante, ya que el fluconazol es un fármaco barato y bien tolerado que se administra fácilmente por vía oral (Wiederhold, 2017). Diversos autores estadounidenses demostraron que la resistencia a fluconazol en *C. glabrata* aumentó en un periodo de diez años (2001-2010) del 18% al 30% (Alexander et al., 2013; Pfaller et al., 2012a). En ciertas áreas geográficas, como Estados Unidos de América y Bélgica, la resistencia global a los azoles en *C. glabrata* teniendo en cuenta, tanto aislados de sangre como de otros sitios anatómicos, fue del 7%-10% (Cleveland et al., 2012; Castanheira et al., 2017; Goemaere et al., 2018) mientras que en otros lugares superaron el 20% (Farmakiotis y Kontoyiannis, 2017), lo que demuestra la variabilidad entre instituciones (Wiederhold, 2017). En España, según el estudio poblacional CANDIPOP (Guinea et al., 2014), la resistencia a fluconazol se encuentra entre un 6-7%, si bien es dependiente de la especie estudiada: *C. albicans* presentó una resistencia del 1,4% frente a *C. glabrata* con una tasa del 9,6%. Esta tasa de resistencia no es muy diferente (9,1%) de la comunicada en un estudio danés realizado en el periodo 2012-2015 (Astvad et al., 2017).

La resistencia a los azoles descansa en distintos mecanismos moleculares, pudiendo ocurrir uno o varios simultáneamente, e implicar resistencias cruzadas entre los distintos azoles (Wiederhold, 2017). En el caso de *C. glabrata,* todos los genes implicados en la vía de síntesis del ergosterol y/o las bombas de eflujo están sobre-expresados en presencia de estos antifúngicos, lo que la convierte en una especie intrínsecamente menos sensible al fluconazol y con facilidad para adquirir resistencia en tratamientos prolongados (Bennett et al., 2004; Rodrigues et al., 2014).

Los azoles actúan inhibiendo la enzima la 14-α-lanosterol desmetilasa, codificada por el gen *ERG11*. Se han descrito más de 140 mutaciones en este gen, pero solo algunas se han correlacionado con la aparición de resistencia a azoles al conferir cambios estructurales en el sitio activo de la enzima (Morio et al., 2010). Además, hay otros genes que podrían modificar la ruta metabólica del ergosterol, entre los que destaca el gen *ERG3*, que codifica una enzima desaturasa de la ruta y su sobre-expresión provoca una disminución del contenido de ergosterol en la membrana y un aumento de metabolitos intermedios tóxicos del ergosterol (Morio et al., 2017). Otras alteraciones en los genes *ERG1*, *ERG6*, *ERG7* y *ERG9* que codifican distintas enzimas de la ruta de la síntesis del ergosterol, también han sido estudiadas y relacionadas con la resistencia a los azoles (Rodrigues et al., 2014). Alternativamente, la sobre-expresión de bombas de eflujo reduce la concentración intracelular de los azoles, y supone la causa más común de resistencia adquirida a los azoles, incluyendo *C. glabrata* (Sanguinetti et al., 2005). Hay dos clases de transportadores implicados, la súper familia de las **A**TP-**b**inding **c**assette (ABC) y la **s**úper familia de las **M**ajor **F**acilitator (MFS). Se han descrito muchos tipos de bombas de eflujo asociadas con la resistencia a azoles y en *C. glabrata* las más frecuentes pertenecen a la familia ABC, entre las que destacan CgCDR1, CgCDR2, CgPDH1 y CgSNQ2 (Sanglard et al., 1999; Vermitsky y Edlind,

2004; Torelli et al., 2008). El factor de transcripción CgPDR1 es el claramente más relacionado con la expresión de estas bombas (Vermitsky et al., 2006; Vale-Silva et al., 2016). Entre los transportadores de la familia de MFS en *C. glabrata*, los más estudiados son CgQdr2 y CgTpo (Costa et al., 2013; Costa et al., 2014).

3.4.4.2. Resistencia a candinas en *C. glabrata*.

El uso de candinas como terapia de primera línea disminuye la mortalidad de los pacientes con candidemia (Andes et al., 2012). Es por esto, que el conocimiento de la resistencia a candinas es particularmente relevante. En estudios de vigilancia, la prevalencia global de la resistencia a candinas en *Candida* spp. es baja, incluso en *C. glabrata* (Pfaller et al., 2013b; Marcos-Zambrano et al., 2014b; Guinea et al., 2014; Klotz et al., 2016; Da Matta et al., 2017; Mencarini et al., 2018). Sin embargo, en ciertas áreas de Estados Unidos de América y del norte de Europa, la resistencia en *C. glabrata* está aumentando (Zimbeck et al., 2010; Alexander et al., 2013; Pfaller et al., 2013b; Pham et al., 2014b; Perlin, 2015b; Vallabhaneni et al., 2015; Astvad et al., 2017). Estudios poblacionales en Estados Unidos de América demuestran que la proporción de aislados de *C. glabrata* causantes de candidemia resistentes aumentaron de un 4,9% a un 12,3% entre 2001 y 2010 y de un 4,2% en 2008 a un 7,8% en 2014 (Alexander et al., 2013; Vallabhaneni et al., 2015), otro estudio también americano mostró tasas cercanas al 10% entre el 2005 y el 2013 (Farmakiotis et al., 2014) (**Figura 10**). En Europa se ha observado también un aumento de la resistencia en los últimos años; en Dinamarca no se registraron aislados resistentes durante el periodo 2004-2007, sin embargo durante el periodo de 2008-2012 se determinó una tasa del 1,4% llegando al 2,7% del 2012 al 2015 (Astvad et al., 2017) (**Figura 10**). Este problema se acrecienta en una especie como *C. glabrata*, ya que se acompaña a menudo de resistencia a azoles (Pfaller et al., 2013b; Alexander et al., 2013; Pham et al., 2014b).

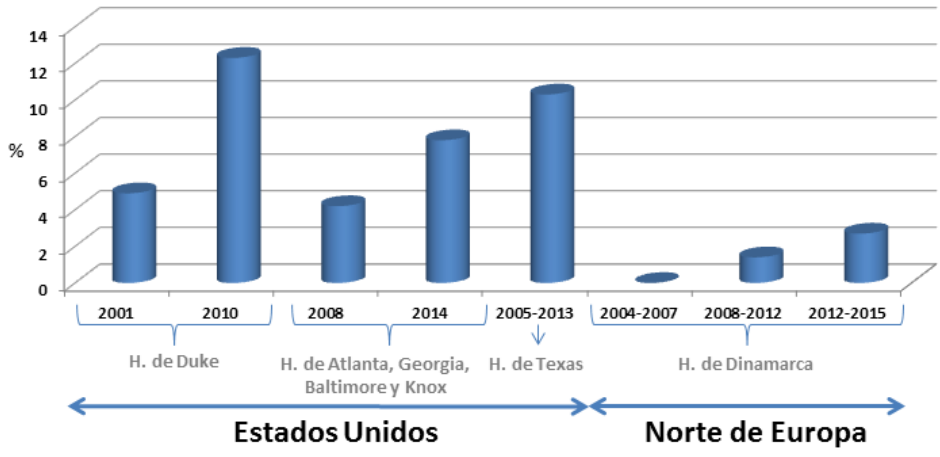

Figura 10. Incremento de la tasa de resistencia a las candinas en *C. glabrata* en los Estados Unidos de América y en el norte de Europa (Alexander et al., 2013; Farmakiotis et al., 2014; Vallabhaneni et al., 2015; Astvad et al., 2017). H; hospital.

Desde una perspectiva clínica este problema podría estar subestimado, debido a que los estudios de vigilancia se centran en aislados de *Candida* spp. causantes de candidemia (Arendrup y Perlin, 2014), o a que los métodos habituales para el diagnóstico de la candidemia y el posterior estudio de la resistencia no sean suficientemente sensibles para la detección de estas cepas resistentes. En un estudio donde se evaluaron cepas de la mucosa oral de pacientes posteriormente tratados con candinas, comparándolos con sus aislados sanguíneos, se detectaron altas tasas de resistencia (cercana al 22%) (Jensen et al., 2015). Del mismo modo, también se encontraron tasas altas de resistencia a las candinas en pacientes con candidiasis abdominal (Shields et al., 2014), llegando a observarse hasta en un 40% de los pacientes que habían sufrido una cirugía o una perforación gastrointestinal, o una pancreatitis necrotizante (Cheng et al., 2014). Un estudio *in vivo* muestra que la resistencia a candinas en *C. glabrata* puede generarse en el tracto gastrointestinal, y de ahí que los mutantes puedan diseminarse a otras localizaciones, sobre todo en los pacientes inmunodeprimidos (Healey et al., 2017). Estos estudios sugieren la existencia de posibles reservorios en el ser humano como fuente de cepas resistentes a candinas.

El único mecanismo en *Candida* spp. de resistencia a las candinas descrito hasta la fecha es la adquisición genética de mutaciones en los genes *FKS* ya que, estos antifúngicos, no son sustratos que utilicen transportadores de otras moléculas. Esto implica la no existencia de resistencia cruzada con los azoles (Niimi et al., 2006; Perlin, 2011).

La presencia de mutaciones en los genes *FKS*, en cualquier especie de *Candida*, provoca una peor respuesta clínica al tratamiento (Shields et al., 2012; Beyda et al., 2014). Sin embargo, se han descrito casos de buena respuesta clínica a las candinas en pacientes infectados con aislados mutantes (Alexander et al., 2013; Beyda et al., 2014), por lo que se necesitan más estudios sobre este tema (Wiederhold, 2016). En candidemias producidas por cepas de *C. glabrata* resistentes a las candinas, se ha llegado a describir unas tasas de fracaso terapéutico de hasta un 80-90%. Además, en infecciones causadas por cepas sensibles en pacientes previamente expuestos a candinas, la respuesta clínica fue del 50% (Shields et al., 2012; Alexander et al., 2013; Shields et al., 2013a; Shields et al., 2013b). Hay autores que recomiendan la búsqueda de mutaciones para la detección de resistencia a candinas ya que puede ser más sensible que la determinación de la CMI (Shields et al., 2012; Wiederhold, 2017).

Las CMIs elevadas, normalmente se correlacionan con sustituciones de aminoácidos en dos regiones específicas bien conservadas dentro de los genes *FKS*, denominados "Hot Spot 1" y "Hot Spot 2", que conllevan una disminución de 50 a 3000 veces en la sensibilidad de la enzima glucano sintetasa al antifúngico, traduciéndose en una menor eficacia *in vivo* (García-Effron et al., 2009b; Arendrup et al., 2012). En *C. glabrata*, además de mutaciones puntuales, se han descrito deleciones y codones de parada tanto dentro como fuera de los "Hot Spot" (García-Effron et al., 2009a; Shields et al., 2012; Alexander et al., 2013; Pham et al., 2014b; Dudiuk et al., 2014) (**Figura 11**).

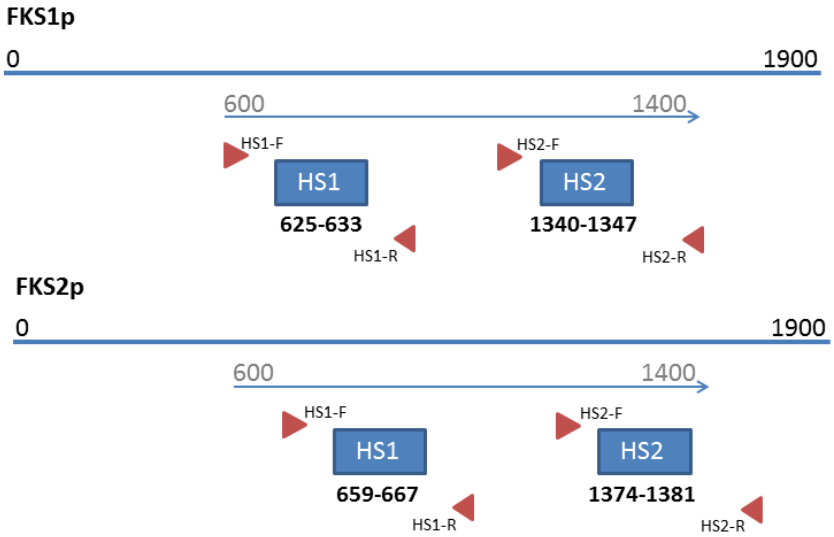

Figura 11. Las regiones conservadas "Hot Spot" (HS) de *C. glabrata* con sus cebadores para amplificar parte del gen *FKS1* y *FKS2*. Adaptado de la literatura (Park et al., 2005; Arendrup y Perlin, 2014).

En la mayoría de los casos se encuentra solamente una mutación en uno de los dos "Hot Spot" en un gen *FKS.* Sin embargo, se han detectado alteraciones fuera de estos "Hot Spot" en los dos genes (*FKS1* y *FKS2*); incluso hay cepas que contienen varias mutaciones a la vez, tanto dentro como fuera de los "Hot Spot" (Castanheira et al., 2014; Pfaller et al., 2014b). En todas las especies de *Candida* estas sustituciones se manifiestan en el gen *FKS1*, exceptuando *C. glabrata,* que también se suelen manifestar en el gen *FKS2* (Perlin, 2007; Katiyar et al., 2012; Arendrup y Perlin, 2014; Arendrup et al., 2014). En la **Tabla 3** se presenta la secuencia aminoacídica de los "Hot Spot" de los genes *FKS1* y *FKS2* de *C. glabrata* y los diferentes grados de resistencia que confiere la sustitución de un determinado aminoácido ya que, se ha demostrado que la CMI de las candinas va a depender de la sustitución del aminoácido y de la especie afectada (Perlin, 2007; García-Effron et al., 2009a; García-Effron et al., 2009b; Arendrup et al., 2011b; Arendrup y Perlin, 2014).

Tabla 3. Secuencias de aminoácidos del "Hot Spot 1" y "Hot Spot 2" de los genes *FKS1* y *FKS2* de *C. glabrata*, indicando las posiciones asociadas con la resistencia (Perlin, 2007; Arendrup y Perlin, 2014).

FKS1		***FKS2***	
"Hot Spot 1"	**"Hot Spot 2"**	**"Hot Spot 1"**	**"Hot Spot 2"**
F*$_{625}$L I L S L R D P	D$_{1340}$W V R R Y T L	F*$_{659}$L I L S L R D P	D$_{1374}$W I R R Y T L

Sustituciones que confieren diferente grado de resistencia: rojo (resistencia fuerte), verde (resistencia débil) y azul (mutación silenciosa); *deleción.

En todas las especies, la elevación de la CMI más significativa se encuentra en el primer y quinto aminoácido (fenilalanina y serina) del "Hot Spot 1" del gen *FKS1* o *FKS2*, considerándolas como alteraciones que confieren una resistencia fuerte a candinas (**Tabla 3**). En cambio, otras sustituciones pueden conferir una resistencia débil con CMIs menos elevadas, pudiendo presentar respuesta al tratamiento con candinas (Perlin, 2011). En *C. glabrata,* las alteraciones más frecuentes se encuentran en el gen *FKS2* (García-Effron et al., 2009a; Katiyar et al., 2012) y la sustitución aminoacídica más frecuente (>50%) que provoca falta de respuesta clínica es la que involucra a la serina de la quinta posición del "Hot Spot 1" del gen *FKS2* (S663) (García-Effron et al., 2009a). Otras alteraciones en el "Hot Spot 1", aunque menos frecuentes, también provocan fallo clínico: S629 en el gen *FKS1* y la F659 en el gen *FKS2* (Cowen et al., 2014).

Se postula que los genes *FKS1* y *FKS2* en *C. glabrata* son funcionalmente redundantes, ya que se pueden producir mutaciones en cualquiera de los dos (Katiyar et al., 2012). Sin embargo, el gen *FKS3* en *C. glabrata* alberga sólo mutaciones silenciosas y se expresan a un bajo nivel en comparación con los demás, por lo que no parece tener influencia en el fenotipo de resistencia (García-Effron et al., 2009a).

En general, los aislados que poseen alteraciones en los genes *FKS* con una elevada CMI a cualquier candina, pueden conferir resistencia cruzada entre ellas, imposibilitando su uso clínico (Arendrup et al., 2014). Sin embargo, se han descrito aislados de *C. glabrata* con mutaciones en los genes *FKS* que tienen diferentes grados de elevación de la CMI, menos elevada a micafungina que a anidulafungina y caspofungina (Arendrup et al., 2011c). Por otra parte, un estudio *in vivo* ha demostrado que aislados de *C. glabrata* con una CMI ligeramente elevada a micafungina responde igual al tratamiento con micafungina que un aislado de tipo salvaje (Arendrup et al., 2012). Además, en *C. glabrata* se observó que la deleción F659 confiere resistencia a las tres candinas y la sustitución F659S, en la misma posición que la alteración anterior, proporciona resistencia solo a dos, anidulafungina y caspofungina, mientras que la CMI de la micafungina permanece sin cambios (Arendrup y Perlin, 2014). Estas observaciones plantean la cuestión de si la micafungina es más eficaz sobre los aislados de *C. glabrata* con algunas mutaciones en el gen *FKS* que las otras dos candinas.

3.4.4.3. Resistencia a anfotericina B en *C. glabrata*.

Se han publicado casos de reducción de la sensibilidad a la anfotericina B en aislados clínicos de *C. glabrata* mediante alteraciones en la vía del ergosterol, que involucran a los genes *ERG2* y *ERG6;* sin embargo, la resistencia adquirida a este antifúngico en las especies de *Candida* es poco frecuente (Vandeputte et al., 2008; Hull et al., 2012). La presencia de mutaciones en el gen *ERG2* da lugar a resistencia cruzada con los azoles (Morio et al., 2017).

3.4.4.4. Multirresistencia en *C. glabrata*.

Las implicaciones clínicas de la multirresistencia en *C. glabrata* son preocupantes, ya que las opciones para tratar este tipo de infecciones son muy limitadas y las guías proporcionan pocas recomendaciones (Cornely et al., 2012; Pappas et al., 2016). La tasa de fracaso clínico aumenta en pacientes con infecciones por *C. glabrata* multirresistentes (Thompson et al., 2008; Lewis et al., 2013) y se sugiere el uso de anfotericina B conjuntamente con un buen control del foco de la infección (Alexander et al., 2013).

Las estrategias para evitar la aparición y la promoción de este tipo de aislados son críticas. Es necesario el control de la fuente de infección (abdominal, dispositivos, etc.) para poder disminuir la carga fúngica y así eliminar las células persistentes que pudieran desarrollar tolerancia a los fármacos con el tiempo (Perlin, 2015a; Perlin, 2015b). Además, los antifúngicos no deben administrarse más tiempo del necesario y se deben administrar dosis que eviten llegar a concentraciones subterapéuticas en el lugar de infección, con la consiguiente promoción de resistencia (Arendrup y Patterson, 2017). La profilaxis, siendo necesaria en pacientes de alto riesgo, debe ser administrada de forma racional, máxime en un momento en el que el número de pacientes expuestos aumentará como consecuencia de la entrada de las formulaciones genéricas de las candinas (Arendrup y Patterson, 2017; Healey y Perlin, 2018). Por último, el uso de fluconazol tanto en el medio hospitalario como en atención primaria, requiere una reflexión como potencial fuente de generación de resistencia.

La definición de multirresistencia para hongos patógenos se ha adaptado de las definiciones establecidas para bacterias por el CDC y ECDC (centro europeo y americano de prevención y control de enfermedades), ya que las opciones terapéuticas para tratar infecciones sistémicas por hongos son más limitadas que en el caso de infecciones bacterianas. En ausencia de una definición estándar para *Candida* spp., la **multirresistencia** se define como un aislado no sensible a uno o más agentes de dos o más clases de antifúngicos, la **multirresistencia extrema** se define como un aislado no sensible a uno o más agentes de tres o más clases de antifúngicos y la **panrresistencia** se define como un aislado no sensible a ningún antifúngico (Arendrup y Patterson, 2017).

Aunque la multirresistencia es poco frecuente (Morio et al., 2017), en los últimos años se ha descrito un aumento específicamente en aislados de *C. glabrata* resistentes a fluconazol y a candinas (Alexander et al., 2013; Perlin, 2015b; Arendrup y Patterson, 2017). En los estudios de vigilancia SENTRY, donde se estudian un número elevado de cepas de *C. glabrata* causantes de candidemia procedentes de distintos centros estadounidenses, no se observó resistencia a candinas ni a fluconazol entre los años 2001 y 2004 (Pfaller et al., 2011b). Sin embargo, entre 2006-2010 un 11% de las cepas de *C. glabrata* que fueron resistentes a fluconazol también lo fueron a una o varias candinas (Pfaller et al., 2012b). Otro estudio estadounidense informó durante el periodo 2001-2010, que un 14% de cepas

de *C. glabrata* resistentes a fluconazol fueron resistentes a una o más candinas (Alexander et al., 2013). Otros autores americanos, tras el análisis de aproximadamente 1.400 aislados de *C. glabrata* durante el periodo 2008-2013, registraron una alta tasa de resistencia, un 36% de los aislados eran resistentes a candinas y a fluconazol (Pham et al., 2014b).

La base genética de la rápida aparición de la resistencia en *C. glabrata* a múltiples antifúngicos sigue siendo desconocida, pero muchos autores sugieren que podría deberse a su estado haploide, ya que le permite adquirir mutaciones de una forma más plástica que a las especies diploides (Arendrup y Perlin, 2014; Perlin, 2014; Shields et al., 2014). Un mecanismo descubierto recientemente en cepas de *C. glabrata* que podría aportar luz a esta cuestión, son los sistemas de reparación del ADN (Healey et al., 2016). Estos sistemas corrigen los errores realizados por la ADN polimerasa, suprimiendo así la aparición de mutantes. Sin embargo, se ha detectado que alteraciones en los genes de estas vías de reparación, por ejemplo en el gen *MSH2*, promueven el desarrollo de resistencia antifúngica, dando lugar a un fenotipo mutador en *C. glabrata* y en diferentes patógenos (Legrand et al., 2007; Healey et al., 2016; Boyce et al., 2017). En *C. glabrata,* según un estudio reciente, las cepas con alteraciones en el gen *MSH2* son significativamente más propensas a producir mutantes de manera más rápida a múltiples fármacos, como el fluconazol, las candinas y la anfotericina B (Healey et al., 2016). Este hallazgo podría ser más relevante en poblaciones donde la profilaxis y el tratamiento con antifúngicos se utiliza de forma más liberal, ya que en estas situaciones se observa una mayor prevalencia de cepas multirresistentes (Healey et al., 2016). Sin embargo, existen discrepancias al respecto ya que, otros autores no encontraron una correlación entre la presencia de mutaciones en el gen *MSH2* y la resistencia antifúngica (Dellière et al., 2016; Hou et al., 2018; Byun et al., 2018; Singh et al., 2018). Cabe destacar que no todas las mutaciones en el gen *MSH2* conducen a un aumento de mutantes *in vitro*, hay ciertos polimorfismos que sí se han asociado con una posible adquisición de resistencia como P208S/N890I, E231G/L269F y V239L, en cambio otros, como E7K, E456D y E459K no se han asociado a resistencia (Healey et al., 2016; Dellière et al., 2016; Singh et al., 2018). Sin embargo, un estudio reciente que utiliza datos de secuenciación del genoma completo en *C. glabrata* sugiere que estas mutaciones en el gen *MSH2* son polimorfismos naturales de este gen (Carreté et al., 2018). Estudios preliminares muestran que otros mecanismos adicionales involucrados en las vías de reparación, como el gen *PMS1* y *MSH6,* son causantes de producir mayores frecuencias de resistencia antifúngica *in vitro* (Healey y Perlin, 2018). Estos defectos en la reparación del ADN podrían ser un mecanismo evolutivo de *C. glabrata* para generar una mayor diversidad genética entre las cepas colonizadoras con el fin de conseguir una mejor adaptación a su entorno, aunque las consecuencias de la diversidad genética en la colonización, infección y resistencia no se conocen exactamente (Healey y Perlin, 2018).

Es posible que otros genes involucrados en la resistencia, o la combinación de varios entren en juego en esta especie, y el gen *MSH2* solo sea una de las piezas dentro de un gran complejo de mecanismos y/o rutas moleculares (**Tabla 4**).

Tabla 4. Genes de *C. glabrata* implicados en la resistencia a antifúngicos. Adaptado de la literatura (Rodrigues et al., 2014).

Gen	Función	Referencias
ERG	Síntesis del ergosterol	(Morio et al., 2010; Rodrigues et al., 2014; Morio et al., 2017)
CDR1-2 *PDH1* *SNQ2* *PDR1*	Transportadores ABC	(Sanglard et al., 1999; Vermitsky y Edlind, 2004; Vermitsky et al., 2006; Torelli et al., 2008; Vale-Silva et al., 2016)
QDR2 *TPO*	Transportadores MFS	(Costa et al., 2013; Costa et al., 2014)
FKS1-2	Síntesis del β-1,3-D-glucano	(Niimi et al., 2006; Perlin, 2011; Shields et al., 2012; Arendrup et al., 2012; Alexander et al., 2013; Shields et al., 2013a; Shields et al., 2013b; Beyda et al., 2014; Wiederhold, 2017)
MSH2	Reparación del ADN	(Healey et al., 2016)
HSP90	Protección estrés celular	(Cowen y Steinbach, 2008; Singh-Babak et al., 2012; Cowen et al., 2014)
EPA	Adhesión y formación de biopelículas	(Cormack et al., 1999; De Las Peñas et al., 2003)
SIR y *RIF*	Formación biopelículas	(Cormack et al., 1999)
BCR1	Regulador de biopelículas	(Gutierrez-Escribano et al., 2012)

Aunque se va avanzando en el estudio de estos mecanismos, todavía hay aspectos sin resolver en la literatura. La mayoría de los estudios están sesgados por el bajo número de aislados de *C. glabrata* invasivos estudiados, por el escaso número de cepas que adquieren resistencia secundaria tanto *in vitro* como *in vivo*, y por la ausencia del correlato clínico y el pronóstico de los pacientes afectados por *C. glabrata* con mutaciones en el gen *MSH2*. Por lo que sería interesante profundizar en estos aspectos.

3.4.4.5. Promoción de resistencia secundaria *in vitro* en *C. glabrata*.

A pesar de que las candinas llevan utilizándose durante años y en la actualidad son de elección para el tratamiento de las infecciones invasivas producidas por *Candida* spp., existen pocos estudios que demuestren el desarrollo de resistencia secundaria *in vitro* a estos antifúngicos (Bartizal et al., 1997; Balashov et al., 2006; Locke et al., 2016; Shields et al., 2018).

En el estudio en el que se testó la actividad de SCY-078 frente a aislados sensibles de *C. glabrata* expuestos a concentraciones crecientes del antifúngico, se observó la aparición de aislados espontáneos con mutaciones en los genes *FKS*, resistentes a este fármaco y a las candinas (Jiménez-Ortigosa et al., 2017). La nueva candina CD101, ha demostrado un bajo potencial de desarrollo de resistencia, similar a las otras candinas (Locke et al., 2016).

Se ha informado de la coexistencia de células sensibles y células resistentes, aunque en menor proporción, fenómeno denominado heterorresistencia. Este hecho puede tener un impacto clínico negativo al comportar una población de células clonales, en la cual las cepas resistentes no son detectadas por las pruebas rutinarias (Healey y Perlin, 2018). La heterorresistencia a fluconazol es bien conocida y puede explicar la mayor tasa de resistencia de *C. glabrata* a fluconazol tras su exposición al fármaco (Ben-Ami et al., 2016).

Existen algunos parámetros específicos adaptados de los antibacterianos para ayudar en la optimización del tratamiento, minimizar la aparición de mutantes y entender el fracaso clínico. Estos conceptos no están bien estudiados en levaduras, si bien podrían ayudar a entender mejor la resistencia en *C. glabrata*. Se trata de la concentración preventiva de mutantes (**m**utant **p**revention **c**oncentration; MPC) y la ventana de selección de mutantes (**m**utant **s**election **w**indow; MSW) (Zhao y Drlica, 2001; Drlica, 2003). La MPC es la concentración de antifúngico donde la probabilidad de encontrar mutantes es baja, por lo que se podría utilizar como medida de sensibilidad ya que, concentraciones por encima de la MPC inhibiría la aparición de resistencia. La MSW es el rango de concentraciones comprendidas entre la CMI y la MPC, donde se promueve o se selecciona la población resistente (Zhao y Drlica, 2001) (**Figura 12** y **Figura 13**).

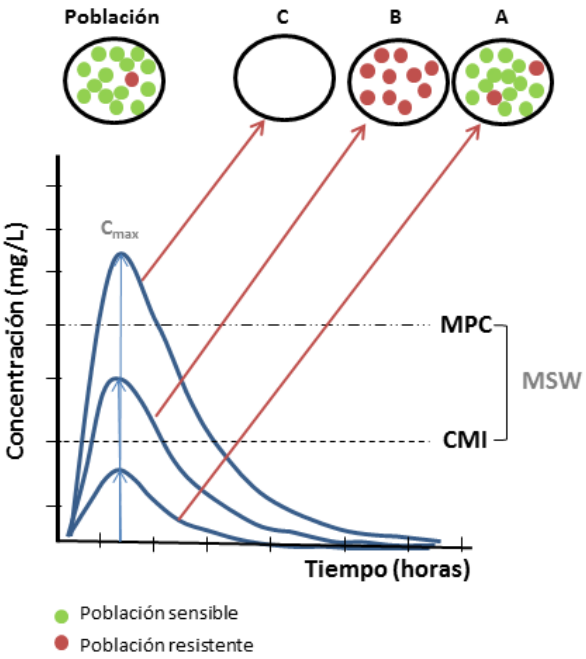

Figura 12. Representación de los parámetros MPC y la MSW y curva farmacocinética del fármaco a lo largo del tiempo. Dependiendo de cuál sea la concentración máxima (C_{max}) alcanzada por el fármaco se observan tres situaciones: **A**, la C_{max} se encuentra por debajo de la CMI por lo tanto, ni se inhibe el crecimiento de la mayoría de las cepas ni apenas se selecciona la subpoblación resistente; **B**, la C_{max} se encuentra en la MSW, por lo que las cepas sensibles son inhibidas y la subpoblación resistente puede ser seleccionada; **C**, la C_{max} es superior a la MPC, donde la inhibición de la población sensible es total y no hay opciones de aparición de mutantes. Adaptado de la literatura (Cantón y Morosini, 2011).

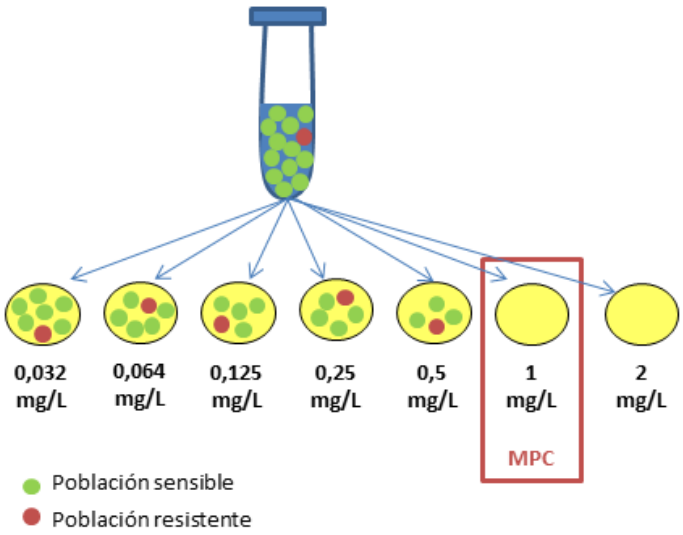

Figura 13. Ejemplo para el cálculo de la MPC de un fármaco dado. Un volumen determinado de un inóculo elevado (10^9-10^{10} UFC/mL) de la cepa a estudiar se siembra directamente en placas de agar con diferentes concentraciones de un fármaco; tras su incubación, la concentración más baja del fármaco donde no se observe crecimiento corresponde a la MPC.

La MPC debe tenerse en cuenta junto con los parámetros farmacocinéticos del antifúngico, ya que las dosis que conduzcan a concentraciones tisulares por encima de la MPC podrían causar toxicidad al paciente y no necesariamente mayor efectividad (Croisier et al., 2004; Firsov et al., 2006; Drlica y Zhao, 2007). Además de lo comentado, la combinación de varios antifúngicos podría ayudar a minimizar la selección de aislados resistentes, tal y como se ha demostrado para los antibacterianos (Cantón y Morosini, 2011).

Los hongos poseen mecanismos adaptativos, entre los que destaca la aparición de tolerancia frente a la presencia de tóxicos celulares, como son los antifúngicos. Entre estos fenómenos, se encuentra el llamado "efecto paradójico" que consiste en el crecimiento de las levaduras en presencia de elevadas concentraciones de candinas (Stevens et al., 2004). Este efecto no está asociado con mecanismos de resistencia a candinas ya que, las cepas que presentan este efecto son susceptibles a estos fármacos; la frecuencia con la que *C. glabrata* muestra este efecto es baja (Stevens et al., 2005; Perlin, 2011; Marcos-Zambrano et al., 2017a). La proteína Hsp90 (**h**eat-**s**hock **p**rotein **90**) y la calcineurina, son dos reguladores críticos a la hora de responder al estrés producido por fármacos (azoles y equinocandinas) (Cowen y Steinbach, 2008; Singh-Babak et al., 2012; Cowen et al., 2014), por lo que su inhibición podría ser una estrategia para mejorar la eficacia de los antifúngicos (Perlin et al., 2015).

3.5. Caracterización genotípica.

Las técnicas de tipado molecular permiten conocer el genotipo de los aislados y consecuentemente la relación genética entre ellos. La caracterización genotípica de los aislados clínicos de *C. glabrata*, pueden ayudar a esclarecer el origen de la infección y detectar la transmisión entre los distintos servicios hospitalarios originando brotes, lo que permite además, mejorar su prevención y control (Escribano et al., 2018). Las técnicas que han demostrado mayor poder de discriminación en *C. glabrata* son el multilocus (**m**ulti**l**ocus **s**equence **t**yping - MLST) y los marcadores microsatélites (**s**imple **t**andem **r**epeats - STR) (Dodgson et al., 2003; Foulet et al., 2005; Grenouillet et al., 2007; Abbes et al., 2012). La técnica de MLST en *C. glabrata* consiste en la secuenciación de seis genes constitutivos o "house-keeping", cuyas secuencias se comparan con bases de datos informatizadas para definir una **s**ecuencia **t**ipo (ST) (Dodgson et al., 2003). Los microsatélites son regiones conservadas, formadas por dos o más nucleótidos (hasta un máximo de seis) repetidos en tándem, distribuyéndose uniformemente a lo largo del genoma. En *C. glabrata* se han diseñado múltiples marcadores, si bien no hay consenso sobre cuáles son los más discriminativos (Foulet et al., 2005; Grenouillet et al., 2007; Brisse et al., 2009; Enache-Angoulvant et al., 2010; Berila y Subik, 2010; Abbes et al., 2012).

Además de la aplicabilidad del genotipado para realizar estudios epidemiológicos, en los últimos años se ha sugerido, en el caso de *C. glabrata*, que existe una asociación entre el genotipo y las alteraciones en el gen *MSH2*, supuestamente relacionadas con la adquisición de resistencia (Dellière et al., 2016; Hou et al., 2018; Byun et al., 2018). Sin embargo se necesitan estudios adicionales, probablemente incluyendo la secuenciación del genoma completo, para confirmar este hecho.

3.6. Patogenicidad de *C. glabrata*: factores de virulencia

Se ha supuesto que las levaduras contribuían pasivamente en el proceso de patogénesis infeccioso. Como consecuencia, la predisposición del paciente inmunodeprimido se consideró el único factor responsable de la infección oportunista. Hoy en día, se considera que el patógeno también juega un papel relevante en la fisiopatología de la enfermedad (Tamura et al., 2007; Silva et al., 2012). La patogenicidad de *Candida* spp. se atribuye a factores de virulencia, como la capacidad de evadir las defensas del huésped mediante formas filamentosas, la capacidad de adherencia, la capacidad de formación de biopelículas (tanto en el huésped como en dispositivos externos) y la producción de enzimas hidrolíticas (Tamura et al., 2007; Silva et al., 2012).

3.6.1. Acondicionamiento físico o "Fitness".

El "fitness" también favorece la capacidad de adaptación y de invasión de las células fúngicas. La virulencia está vinculada a la capacidad de dividirse y por tanto al crecimiento, estudiado a través de los parámetros cinéticos mediante curvas de densidad óptica *in vitro*, especialmente la velocidad de crecimiento. Además, las curvas de crecimiento permiten la medición de parámetros cinéticos sugiriendo alteraciones en el "fitness" entre las diferentes especies de *Candida* (Ben-Ami et al., 2011). Algunas cepas clínicas de *C. albicans* resistentes a las candinas (S645P o S645F) presentan peor "fitness" ya que, su velocidad de crecimiento es menor que la de sus respectivas cepas salvajes (Ben-Ami et al., 2011). A día de hoy, este sigue siendo aún un tema controvertido y son necesarios estudios de parámetros cinéticos con un mayor número de cepas sensibles y resistentes a los antifúngicos.

3.6.2. Modelos animales para el estudio de la virulencia.

Distintos modelos animales se han empleado para estudiar la función del sistema inmune del huésped a la hora de combatir las infecciones por *Candida* spp., contribuyendo al mejor conocimiento de la interacción patógeno-hospedador. Los modelos de referencia son los animales vertebrados, concretamente el modelo murino. Sin embargo, este modelo tiene desventajas, como el alto coste y unas cuestiones éticas relacionadas con su manipulación, por lo que se han buscado alternativas en los últimos años. El modelo invertebrado de *Galleria mellonella*, (Lepidóptera: Pyralidae, gusano de la miel) es el que proporciona más beneficios, en comparación con otros modelos, para el estudio de la virulencia en cepas de *Candida* spp. (Borghi et al., 2014b; Marcos-Zambrano et al., 2017c). Diversas características del modelo, como las siguientes, lo convierten en un gran candidato para el estudio de la infección en *Candida* spp. Es un método de fácil manipulación y económico, lo que permite un ensayo con un gran número de larvas y por consiguiente un aumento del poder estadístico. Tiene una alta correlación con los modelos vertebrados ya que, su respuesta inmunitaria es muy parecida a la de los mamíferos. El inóculo de infección

es fácil de controlar, por ello es sencilla la monitorización de la infección. Además, la incubación de la larva es posible a 37 °C, lo que permite el estudio de la virulencia a la temperatura del cuerpo humano (Brennan et al., 2002; Fuchs et al., 2010; Browne et al., 2013; Binder et al., 2016; Frenkel et al., 2016; Wojda, 2017; Ames et al., 2017). El modelo de *G. mellonella* actualmente se ha utilizado para correlacionar la producción de biopelícula *in vivo*/*in vitro* (Borghi et al., 2014b; Rajendran et al., 2015a).

3.6.3. Biopelículas.

La biopelícula fúngica es una estructura biológica ordenada, tridimensional, dinámica y compleja que consiste en una red de células adheridas en el interior de una matriz extracelular de lípidos, polisacáridos, proteínas y ácidos nucleicos (D'Enfert, 2006; Perlin et al., 2015) y esta matriz es la que las distingue de las células planctónicas, aportándole numerosas ventajas (Cowen et al., 2014). Las levaduras forman biopelículas fácilmente bajo una amplia variedad de factores ambientales [pH, temperatura, osmolaridad, la presencia de bacterias, el medio circundante (orina, sangre, saliva y mucosidad), la presencia de fármacos e inmunidad del huésped] (Kumamoto, 2002; Rodrigues et al., 2014). De hecho, pueden ser responsable de hasta el 65% de todas las infecciones microbianas humanas (Mah y O'Toole, 2001). La capacidad de formar biopelículas puede conferir en *Candida* spp. una ventaja de supervivencia ya que, ayuda a evadir los mecanismos inmunitarios del huésped y a resistir el tratamiento antifúngico y la presión competitiva de otros microorganismos. Además, puede ser responsable de la buena adaptación a la colonización de tejidos y dispositivos externos, a pesar de que por ejemplo, *C. glabrata* no es capaz de formar filamentos (Silva et al., 2010; Rodrigues et al., 2014).

La matriz extracelular en *Candida* spp. está compuesta mayoritariamente por 1,3-β-D-glucano, que es capaz de secuestrar a los antifúngicos, disminuyendo su concentración efectiva a nivel de la membrana y provocando que la biopelícula sea difícil de erradicar (Mitchell et al., 2013). Estas condiciones podrían favorecer la selección de aislados resistentes, actuando como reservorios y aumentando la probabilidad de entrar en el torrente circulatorio y causar infecciones sistémicas (Perlin, 2014; Perlin, 2015a). Diferentes mecanismos pueden ser responsables de la resistencia de las biopelículas en *C. glabrata*, como la alta densidad de células dentro de la biopelícula, la disminución de la tasa de crecimiento, la limitación de nutrientes, la presencia de fenotipos variables conocidas como células persistentes o latentes y la expresión de genes de resistencia, tanto de bombas de eflujo como modificaciones en la vía de biosíntesis del ergosterol (Rodrigues et al., 2014; Morio et al., 2017). La densidad celular es un factor importante para la resistencia antifúngica ya que, en las biopelículas densas, existe una cooperación entre las células denominado "quorum sensing", que permite a la matriz organizarse, comunicarse y coordinar su comportamiento mediante la secreción de moléculas de señalización (Ramage et al., 2012). Las biopelículas de *Candida* spp. son intrínsecamente resistentes a los azoles,

involucrando mecanismos multifactoriales, como la inducción de bombas de eflujo y el secuestro del fármaco dentro de la estructura extensa de la matriz (Kumamoto, 2002; Mukherjee et al., 2003; Ramage et al., 2009). Sin embargo, las candinas que inhiben la síntesis de glucano componente fundamental de la matriz extracelular, junto a la anfotericina B, poseen actividad anti-biopelícula (Kuhn et al., 2002; Perlin, 2011).

La biomasa y la viabilidad de las biopelículas pueden cuantificarse mediante diferentes métodos microbiológicos, químicos, físicos, moleculares y microscópicos (Azeredo et al., 2017). Los métodos metabólicos son los mejores candidatos para la cuantificación de la viabilidad de las células ya que, sin requerir mucha manipulación de la muestra se pueden detectar productos metabólicos producidos por ellas. Es un método indirecto, por lo que se necesitan indicadores para la detección de la actividad metabólica, entre los que destaca la sal de tetrazolio (XTT), que mediante su reducción por parte de las células metabólicamente activas, da lugar a un producto que se puede medir espectrofotométricamente (Pierce et al., 2008). Otro método para cuantificar la biopelícula es el estudio de la biomasa, mediante la cuantificación del grosor y la estructura de la matriz. El método más común es la tinción con cristal violeta, que consta de una medición de forma indirecta de la adsorción de este colorante por parte de las células que conforman la biopelícula (O'Toole, 2011; Azeredo et al., 2017). Ambos parámetros son complementarios ya que, la medición del XTT aporta una idea de viabilidad de las células y el cristal violeta da una idea del grosor de la biopelícula (Marcos-Zambrano et al., 2014a).

Se sabe que no todas las especies de *Candida* tienen la misma capacidad de producir biopelículas, existiendo diferencias tato inter como intra-especie (Marcos-Zambrano et al., 2014a). Las biopelículas de *C. glabrata* muestran la mayor actividad metabólica en comparación con otras especies de *Candida* ya que, tienen cantidades más altas de proteínas y carbohidratos (en algunos casos, cinco veces mayor); por contra, es la especie con menor producción de biomasa (Silva et al., 2010; Rodrigues et al., 2014; Marcos-Zambrano et al., 2014a).

Se necesitan estudios adicionales sobre la patogenicidad de estos mutantes *FKS* de *C. glabrata* (capacidad para producir biopelículas, curvas de crecimiento y virulencia), en comparación con los aislados salvajes.

4. JUSTIFICACIÓN

La infección fúngica invasora, concretamente la candidemia, ha aumentado significativamente en las últimas décadas y junto a su alta morbi-mortalidad, hace necesario el estudio exhaustivo de las características tanto de los pacientes infectados como de los patógenos que la causan.

El tratamiento de elección para estas infecciones son los azoles y las candinas, siendo estas últimas las recomendadas como primera elección por presentar mayor eficacia clínica y por su mejor perfil farmacocinético. La especie más frecuente y más sensible a los antifúngicos es *C. albicans*. Sin embargo, desde hace algún tiempo, la epidemiología de la candidemia está cambiando y la incidencia de *C. glabrata* está aumentando notoriamente, incluso a costa de *C. albicans*. Además, en ciertas áreas geográficas a ambos lados del océano Atlántico, se está describiendo un aumento significativo de la tasa de resistencia a candinas en esta especie provocando una alerta clínica ya que, imposibilita instaurar el tratamiento de primera línea y añade complicación al manejo terapéutico por su intrínseca sensibilidad reducida a los azoles. Recientemente, se ha demostrado que algunos santuarios biológicos, como el abdomen o las mucosas, donde las candinas no llegan a alcanzar concentraciones terapéuticas adecuadas, pueden actuar como reservorios ocultos de cepas de *C. glabrata* resistentes a estos antifúngicos. En España, la tasa de resistencia a candinas en *C. glabrata* comunicada hasta la fecha es baja y aunque no parece que vaya en aumento, la presión antifúngica sobre un patógeno proclive a desarrollar resistencia secundaria supone una continua amenaza en un momento en el que las candinas pasen a ser fármacos genéricos, cuyo uso seguramente aumentará.

Existen algunas carencias en la literatura médica sobre el tema planteado. Se requieren estudios que clarifiquen: i) el desarrollo de resistencia secundaria a equinocandinas en *C. glabrata* tras exposición a estos fármacos *in vitro* y los mecanismos moleculares que puedan estar involucrados en la adquisición de resistencia; ii) estudios de los parámetros cinéticos de crecimiento en cepas de *C. glabrata* salvajes y resistentes; iii) evaluación de técnicas rápidas de detección de resistencia a candinas. En esta tesis se ha pretendido dar luz a estos interrogantes.

5. OBJETIVOS

La siguiente tesis se organiza en tres capítulos, cuyos objetivos concretos se listan a continuación:

Capítulo I: Adquisición *in vitro* de resistencia secundaria a equinocandinas en aislados de *C. glabrata* causantes de candidemia y mecanismos moleculares implicados.

1. Estudiar la capacidad de cepas de *C. glabrata* para adquirir resistencia secundaria a equinocandinas tras exposición *in vitro* a concentraciones crecientes y constantes de micafungina.
2. Calcular la concentración preventiva de mutantes, la ventana de selección de mutantes y la frecuencia de mutación a micafungina y anidulafungina en cepas de *C. glabrata*.
3. Comparar los parámetros cinéticos de crecimiento y la virulencia entre cepas salvajes y cepas resistentes a las equinocandinas de *C. glabrata* productoras de candidemia.
4. Evaluar la relación entre la secuencia del gen *MSH2* en cepas de *C. glabrata* causantes de candidemia con el genotipo obtenido mediante dos técnicas de genotipado (microsatélites y MLST), la posible adquisición de resistencia secundaria *in vitro* e *in vivo*, y el pronóstico del paciente.

Capítulo II: Determinación de parámetros cinéticos de crecimiento *in vitro* en aislados de *Candida* spp. causantes de candidemia.

5. Determinar los parámetros cinéticos de crecimiento en aislados clínicos de diferentes especies de *Candida* para estudiar diferencias inter-especie, intra-especie, y entre cepas de *C. glabrata* sensibles y resistentes a equinocandinas.
6. Análisis de la cinética de crecimiento de *Candida* spp. a lo largo del tiempo de incubación con objeto de adelantar la obtención de los resultados de sensibilidad antifúngica.

Capítulo III: Detección rápida de resistencia a equinocandinas en *Candida* spp.

7. Evaluar el papel del Etest® y de las placas de agar con anidulafungina realizados directamente sobre hemocultivos, para la detección de resistencia a las equinocandinas en *Candida* spp., y específicamente en situaciones de coexistencia de cepas de *C. glabrata* sensibles y resistentes.

6. DESARROLLO

6.1. CAPÍTULO I: Adquisición *in vitro* de resistencia secundaria a equinocandinas en aislados de *C. glabrata* causantes de candidemia y mecanismos moleculares implicados.

6.1.1. Artículo 1: *In vitro* exposure to increasing micafungin concentrations easily promotes echinocandin resistance in *Candida glabrata* isolates.

El objetivo de este estudio fue evaluar la capacidad de *C. glabrata* para desarrollar resistencia secundaria a las equinocandinas tras la exposición *in vitro* a concentraciones crecientes de micafungina.

Cinco aislados de *C. glabrata* sensibles a las equinocandinas se expusieron a diferentes concentraciones de micafungina (desde 0,031 mg/L hasta 2 mg/L) sobre placas de agar. La sensibilidad antifúngica determinada mediante EUCAST (micafungina, anidulafungina y fluconazol) y la secuencia de los genes *FKS* fueron estudiadas tras la exposición sobre placas con concentraciones crecientes de micafungina (exposición progresiva) o la siembra directa en placas con diversas concentraciones de fármaco.

La exposición progresiva condujo a elevaciones de las CMIs de micafungina y anidulafungina a medida que se aumentaron las concentraciones de micafungina en las placas. Todos los aislados se volvieron resistentes a una o ambas equinocandinas en presencia de bajas concentraciones de micafungina (0,062 mg/L o 0,125 mg/L) y adquirieron mutaciones en el gen *FKS2*. Sin embargo, la CMI de fluconazol permaneció sistemáticamente inalterada. Por el contrario, la exposición directa a micafungina produjo una inhibición del crecimiento en placas con concentraciones de micafungina ≥0,062 mg/L tras 24 horas de incubación.

Se concluyó que una exposición progresiva a concentraciones crecientes de micafungina *in vitro* promovió fácilmente resistencia a las equinocandinas en aislados clínicos susceptibles de *C. glabrata* causantes de candidemia, incluso a una baja concentración de micafungina. Por el contrario la exposición directa a ciertas concentraciones de equinocandinas no permitió la adquisición de resistencia.

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In Vitro Exposure to Increasing Micafungin Concentrations Easily Promotes Echinocandin Resistance in *Candida glabrata* Isolates

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ABSTRACT We assessed the *in vitro* susceptibility of five echinocandin-susceptible *Candida glabrata* isolates after exposure to micafungin. The direct exposure to plates at different micafungin concentrations resulted in the inhibition of growth at 0.062 μg/ml. The progressive exposure was performed on plates using 0.031 μg/ml of micafungin and sequential propagation on plates containing the next 2-fold concentration; the MICs of micafungin and anidulafungin increased sequentially, and all the isolates became echinocandin resistant, showing *fks2* mutations.

KEYWORDS echinocandin, micafungin, *fks* mutation, *Candida glabrata*, FKS

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Echinocandins are currently recommended as the first-line treatment for invasive candidiasis (1–3). Although the resistance to echinocandins reported in Spain remains low (4), emerging *Candida glabrata* echinocandin-resistant isolates have been reported elsewhere (5, 6). We hypothesized that, as in the case of *Aspergillus fumigatus* (7), the *in vitro* exposure to increasing concentrations of echinocandins could promote the generation of mutations conferring resistance.

(This study was partially presented at the 26th European Congress of Clinical Microbiology and Infectious Diseases in Amsterdam, The Netherlands, 2016 [8].)

C. glabrata isolates from five patients with candidemia admitted to Ramón y Cajal Hospital (Madrid) were initially tested for antifungal susceptibility to micafungin (Astellas Pharma, Inc., Tokyo, Japan), anidulafungin, and fluconazole (Pfizer Pharmaceutical Group, New York, NY, USA) according to the EUCAST EDef 7.2 microdilution procedure ($MIC_{initial}$) (9–12).

The isolates were grown on chromogenic agar plates and incubated at 35°C for 24 h to check for purity. A loopful of cultured isolates was suspended in 10 ml of yeast extract-peptone-dextrose (YPD) broth (Becton Dickinson, Madrid, Spain) and incubated at 30°C overnight with vigorous shaking (150 to 160 × *g*) in an orbital shaker. Yeast cells were collected by centrifugation (3,000 × *g* for 5 min), and the pellet was resuspended in phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK) and centrifuged again for washing. Washed suspensions were adjusted to 2×10^9 to 4×10^9 (mean, $2.94 \pm 0.89 \times 10^9$) CFU/ml using a Neubauer chamber and stroked on micafungin-containing plates under two different sets of conditions (direct exposure and progressive exposure). Sabouraud dextrose agar plates were prepared using eight different micafungin concentrations (0.015, 0.031, 0.062, 0.125, 0.25, 0.5, 1, and 2 μg/ml) for the exposure

TABLE 1 Susceptibilities of *C. glabrata* isolates to micafungin and anidulafungin before and after progressive exposure to micafungin

	MIC (μg/ml)			
	Micafungin		Anidulafungin	
Exposure[a]	Range	Geometric mean	Range	Geometric mean
Initial	0.015	0.015	0.062	0.062
Progressive				
Subsequent	0.015–2	0.18[b]	0.031–4	0.56[b]
Final	0.062–4	1.15[c]	1–4	2.30[c]

[a]Initial, before progressive exposure to micafungin; Subsequent and Final, after exposure to micafungin.
[b]Data represent results of comparisons between the geometric means of the initial MIC and the subsequent MIC, for which significant differences were determined ($P < 0.05$).
[c]Data represent results of comparisons between the geometric means of the initial MIC and the final MIC, for which significant differences were determined ($P < 0.05$).

experiments, all of which were set up in duplicate to ensure reproducibility. In the direct micafungin exposure experiments, adjusted inocula (100 μl) were directly transferred to plates containing the eight different micafungin concentrations and the plates were incubated at 35°C and visually inspected for growth after 24 h. The MICs of micafungin and anidulafungin against the isolates growing on the plates containing the highest micafungin concentration were determined. In the progressive micafungin exposure experiments, adjusted inocula (100 μl) were stroked on plates containing micafungin at 0.031 μg/ml and the plates were incubated at 35°C for 24 h. If growth was observed, a loopful of cultured isolates was spread on the plate with a concentration of micafungin that was 2-fold greater. These steps were repeated up to the concentration of 2 μg/ml of micafungin. Each sequential suspension was used to study the MIC of micafungin and anidulafungin at each subsequent propagation step ($MIC_{subsequent}$) and final propagation step (MIC_{final}), and echinocandin and fluconazole MICs were determined using the plates containing 2 μg/ml of micafungin with the aid of EUCAST. Geometric means of $MIC_{initial}$, $MIC_{subsequent}$, and MIC_{final} of micafungin and anidulafungin were compared using the Wilcoxon signed-rank test (PASW Statistics 18.0; SPSS Inc., Chicago, IL). The comparisons were considered statistically significant with a *P* value of <0.05.

fks1 and *fks2* genes from the isolates used to study the $MIC_{initial}$, $MIC_{subsequent}$, and MIC_{final} were amplified (13, 14). The stability of phenotypic and genotypic resistance was studied.

The isolates were genetically unrelated, fluconazole intermediate, echinocandin susceptible ($MIC_{initial}$), and wild type (Table 1). The direct micafungin exposure allowed all isolates to grow on plates containing micafungin at concentrations up to 0.031 μg/ml, but the echinocandin MICs studied in these isolates were identical to $MIC_{initial}$. The exposure to progressively increasing concentrations of micafungin allowed isolates to grow on all micafungin-containing plates. Both $MIC_{subsequent}$ and MIC_{final} were significantly higher than the $MIC_{initial}$ (Table 1). Overall, a trend toward higher echinocandin MICs was observed with increasing micafungin concentrations in the plates but with stable MICs of fluconazole. All isolates grown on plates with micafungin at 0.062 μg/ml or 0.125 μg/ml became resistant to anidulafungin and/or micafungin, but two relevant observations were made (Table 2). First, at 0.125 μg/ml, four isolates were micafungin and anidulafungin resistant and *fks2* mutations were found; the remaining isolate (CG3) was resistant only to anidulafungin but had the wild-type *fks2* gene. However, the MIC of anidulafungin against the CG3 isolate rose progressively and a deletion at F658 in *fks2* was found when the isolate was grown on 0.25 μg/ml micafungin plates, although micafungin resistance was detected only in the last plate. Second, the CG1 isolate was anidulafungin and micafungin resistant on plates containing micafungin at 0.062 μg/ml, and two mutations (D666Y and S663P) in the *fks2* gene were found; however, only the S663P substitution was found when isolates were grown

TABLE 2 Antifungal activity of micafungin and anidulafungin against the five isolates before and after progressive micafungin exposure[a]

Exposure	Result(s) for indicated isolate									
	CG1		CG2		CG3		CG4		CG5	
	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation	EUCAST MIC (μg/ml) (MYC/AND)	*fks2* mutation
Initial	0.015/0.062	WT	0.015/0.062	WT	0.015/0.062	WT	0.015/0.062	WT	0.015/0.062	WT
Subsequent (MYC concn in plates, μg/ml)										
0.031	0.015/0.031	WT	0.015/0.062	WT	0.015/0.062	WT	0.015/0.031	WT	0.015/0.031	WT
0.062	**0.5/2**	**S663P D666Y**	0.015/0.062	WT	0.015/**0.125**	WT	0.015/0.062	WT	0.015/0.062	WT
0.125	**1/2**	**S663P**	**0.125/0.5**	**S663Y**	0.031/**0.25**	WT	**2/2**	**Del F658**	**2/2**	**Del F658**
0.25	**1/2**	**S663P**	**0.125/1**	**S663Y**	0.031/**0.5**	**Del F658**	**2/2**	**Del F658**	**2/2**	**Del F658**
0.5	**2/2**	**S663P**	**0.25/2**	**S663Y**	0.031/**1**	**Del F658**	**2/2**	**Del F658**	**2/2**	**Del F658**
1	**2/4**	**S663P**	**0.5/2**	**S663Y**	0.031/**1**	**Del F658**	**2/2**	**Del F658**	**2/4**	**Del F658**
2	**4/4**	**S663P**	**1/2**	**S663Y**	**0.062/1**	**Del F658**	**2/2**	**Del F658**	**4/4**	**Del F658**
Subsequent geometric mean of MICs	0.74/1.34		0.12/0.55		0.03/0.37		0.49/0.67		0.55/0.82	

[a]MYC, micafungin; AND, anidulafungin; Del, deletion. Boldface characters represent MICs above the clinical breakpoints and FKS point mutations.

on the plates with the subsequent concentration of micafungin. The *fks* substitutions and the phenotypic resistance were stable and reproducible after several propagations on micafungin-free agar plates. Genotyping showed that isolates were identical before and after micafungin progressive exposure, thus excluding any potential contamination of the isolates during the propagation steps.

The emergence of echinocandin resistance could be caused by the predisposition of this pathogen to easily acquire mutations in response to drug pressure due to its haploid nature and to alterations caused by mismatched repair genes (15). The five isolates studied became echinocandin resistant when grown even on plates containing low concentrations of micafungin. Micafungin penetrates slightly in the peritoneal fluid; the reported peritoneal fluid/plasma ratio (area under the concentration-time curve from 0 to 24 h [AUC_{0-24}]) is 0.3 (16). Shields et al. hypothesized that the abdomen of patients with previous exposure to echinocandins can be a hidden reservoir for mutant-resistant *C. glabrata* isolates (6). This suggests that the exposure to low echinocandin concentrations may promote the generation of mutant isolates that may potentially cause invasive infections. In our *in vitro* study, resistance was obtained at concentrations close to the MIC.

No resistant isolates were obtained with direct exposure. On the other hand, mutations were found in the progressive exposure experiment with the same isolates, suggesting that increasing micafungin concentrations may be effective in terms of selecting and enriching underrepresented mutant populations. In this sense, the CG1 isolate illustrates the phenomenon of coexistence of several populations, as two mutations were detected when the isolate was grown on plates containing low micafungin concentrations. The D666Y mutation confers weak resistance, while the S663P mutation confers strong resistance (17). However, only the S663P mutation was found when the isolate was grown on plates containing higher micafungin concentrations. CG3 became resistant to anidulafungin, and this isolate also showed micafungin resistance in the last step of progressive exposure. Some isolates can be resistant to anidulafungin and susceptible to micafungin (18). This supports the use of anidulafungin as a surrogate marker to predict echinocandin resistance. Furthermore, the deletion at position F658 does not correlate with a predictable pattern of susceptibility to candins.

Echinocandin resistance in *C. glabrata* has been associated with a loss of fitness in isolates with mutations at S663P in the *fks2* gene (17). The fitness cost for the isolates carrying a mutation could explain the relatively low spread of resistant isolates reported to date. Further studies should be done on this topic.

This study had limitations. Despite of the low number of isolates analyzed, this proof of concept would be enhanced by the inclusion of a large number of clinical isolates. This was an *in vitro* study, and its impact in clinical practice is unknown, although our observations help improve understanding of the previous clinical reports on the presence of *C. glabrata* mutant isolates sourced from anatomical sites with low echinocandin concentrations. In conclusion, we found that a progressive exposure to increasing concentrations of micafungin can easily promote resistance to echinocandins in *C. glabrata* clinical isolates.

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6.1.2. Artículo 2: Low and constant micafungin concentrations may be sufficient to lead to resistance mutations in *FKS2* gene of *Candida glabrata*.

En este trabajo se estudió la capacidad de las cepas de *C. glabrata* productoras de candidemia para adquirir resistencia secundaria a las equinocandinas tras una exposición *in vitro* a concentraciones bajas y constantes de micafungina. Se caracterizó la cinética de crecimiento y la virulencia de los mutantes obtenidos.

Cinco aislados de *C. glabrata*, se expusieron a concentraciones bajas y constantes de micafungina (0,031 mg/L) tras pases diarios sucesivos (hasta 10 días). Tras cada pase se estudió la sensibilidad antifúngica (micafungina, anidulafungina y fluconazol) mediante EUCAST, y se caracterizó la secuencia de los genes *FKS*. Al décimo día de exposición se evaluó la patogenicidad de las cepas (parámetros cinéticos de crecimiento y virulencia en el modelo *in vivo* de *Galleria mellonella*) y se estudió la secuencia del gen *MSH2*.

Todos los aislados adquirieron resistencia fenotípica a equinocandinas y mutaciones en el gen *FKS2* tras 2-4 días de exposición. Sin embargo, la CMI de fluconazol no sufrió grandes cambios. Las cepas no necesariamente provenían de pacientes que previamente recibieran tratamiento antifúngico, no presentaron mutaciones específicas en el gen *MSH2*, ni mostraron cambios en los parámetros cinéticos de crecimiento. Sin embargo, los aislados con mutaciones en el gen *FKS2* mostraron menor virulencia que las cepas salvajes en el modelo de *G. mellonella*.

Se concluyó que las cepas de *C. glabrata* adquirieron resistencia a equinocandinas rápidamente cuando fueron expuestas *in vitro* a concentraciones bajas y constantes de micafungina. La adquisición de mutaciones en los genes *FKS* parece que puede afectar a la virulencia de las cepas de *C. glabrata*.

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Brief Report

Brief Report

Low and constant micafungin concentrations may be sufficient to lead to resistance mutations in *FKS2* gene of *Candida glabrata*

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Abstract

We studied the ability of five echinocandin-susceptible *C. glabrata* isolates to acquire *in vitro* resistance to anidulafungin and micafungin. All isolates became phenotypically resistant after 2–4 days of exposure to low and constant micafungin concentrations ($P < .05$). Mutations in the HS1 region of the *FKS2* gene were found in all isolates. The acquisition of resistance was not related to the previous use of antifungal treatment in the patients or the presence of mutations at *MSH2* gene. We found differences ($P < .0001$) in the median survival of *Galleria mellonella* larvae infected with *FKS2* mutant isolates (5 days) and wild-type isolates (3 days).

Key words: echinocandins, micafungin, FKS mutation, *Candida glabrata*, *Galleria mellonella*.

The number of cases of candidemia caused by *Candida glabrata* is on the rise.[1] *C. glabrata* shows intrinsically low susceptibility to azoles, and emerging resistance to echinocandins has been reported in isolates from patients on long-term therapy.[2,3] Resistant isolates show elevated echinocandin MICs and harbor mutations in *FKS* genes.[4]

We previously reported five wild-type, genetically unrelated using microsatellite typing, and echinocandin-susceptible *C. glabrata* isolates from five patients with candidemia admitted to Ramón y Cajal Hospital (Madrid, Spain) that became resistant after *in vitro* exposure to increasing micafungin concentrations.[5] Here, we report on

the ability of the same set of isolates to acquire resistance after exposure to a low and constant concentration of micafungin (0.031 mg/l). Furthermore, the fitness and virulence of the resulting *FKS* mutations were studied. Isolates were tested for antifungal susceptibility to micafungin (Astellas Pharma, Inc, Tokyo, Japan), anidulafungin, and fluconazole (Pfizer Pharmaceutical Group, New York, NY, USA) according to the EUCAST EDef 7.2 microdilution procedure ($MIC_{initial}$).[6] The procedure used to study the development of resistance was previously reported with some modifications.[5] The isolates were grown on chromogenic agar plates and incubated at 35°C for 24 h. A loopful of cultured isolates was suspended in 10 ml of YPD broth (Becton Dickinson, Madrid, Spain) and incubated at 30°C overnight with vigorous shaking (150–160 *g*) in an orbital shaker. Yeast cells were collected by centrifugation (3000 *g* for 5 minutes), and the pellet was resuspended in phosphate-buffered saline (PBS) (Oxoid, Hampshire, UK) and centrifuged again for washing. Washed suspensions were adjusted to 4 to 6×10^9 (mean of $4.7 \pm 0.98 \times 10^9$) using a Neubauer chamber and 100 μl of the suspensions were streaked on plates containing micafungin (0.031 mg/l), which were then incubated for 24 hours at 35°C. If growth was observed, an adjusted suspension prepared using the whole slime found on the plate surface; 100 μl of the suspension were propagated on another plate containing micafungin at the same concentration. This suspension was used to study the MIC of micafungin, anidulafungin, and fluconazole following the EUCAST procedure[6] and to sequence the genes *FKS1* and *FKS2*.[7] The procedure involved 10 propagation steps. The geometric means of $MIC_{initial}$ and MIC_{final} (last propagation step) of micafungin and anidulafungin were studied and compared (Wilcoxon signed-rank test, IBM SPSS Statistics for Windows, Version 21.0, IBM Corp, Chicago, IL, USA). *MSH2* gene sequences[8] and the *in vitro* growth kinetics parameters (average growth rate and time to maximum rate)[9] were studied in wild-type and mutant isolates obtained after the last propagation step.

After the last propagation step, we compared the mortality caused by wild-type isolates and by isolates harboring *FKS* mutations in final instar larvae of *G. mellonella* (Bichosa, Salceda de Caselas, Spain). We used an infecting inoculum (5×10^6 cfu per larva) measured with a Neubauer chamber able to generate 50% larva mortality 96 hours after infection.[10] Inocula ranging from 3 to 7×10^6 cfu per larva were accepted because the inoculum ranges did not affect the mortality of the larvae. Ten *G. mellonella* larvae per isolate were infected with 10 μl of a suspension of *C. glabrata*, and two control groups were established, one comprising 10 larvae inoculated with 10 μl of PBS to monitor trauma and another comprising 10 noninjected larvae. Each experimental group contained randomly chosen larvae of an appropriate weight (330 ± 20 mg). Larvae were incubated at 37°C for up to 7 days after infection, and the number of dead larvae was recorded daily.[10] Survival curves were obtained for wild-type isolates and isolates harboring *FKS* mutations using the Kaplan–Meier method (Graph Pad Prism statistical 5.02 software, GraphPad, La Jolla, CA, USA), and differences were evaluated (log-rank test and Mantel-Cox test).

This study was approved by the Ethics Committee of Hospital Gregorio Marañón (CEIC-A1; study no. 208/16).

Resistance to echinocandins is increasingly reported in *C. glabrata*, although not in other *Candida* spp., thus indicating that *C. glabrata* has the potential to acquire resistance to echinocandins.[2,4] The isolates studied here became phenotypically resistant to echinocandins after 2–4 days of exposure to micafungin (Table 1). Once an isolate became resistant, the MIC was invariably high at all the propagation steps. The geometric mean of the micafungin and anidulafungin MIC_{final} was significantly higher than that of the $MIC_{initial}$ ($P < .05$). In contrast, the MIC of fluconazole did not increase in any of the isolates (data not shown). Phenotypically resistant isolates harbored mutations in the HS1 region of the *FKS2* gene (Table 1). The CG2 isolate harbored a substitution at W715L (outside the HS1 region) that had not been previously reported; the previous mutations have been previously found in clinical isolates. *FKS* mutations and phenotypic resistance were stable after five propagations on micafungin-free agar plates. Recent reports showed that the presence of a *MSH2* mutator genotype and/or exposure to antifungals[8,11] may contribute to the development of resistance to fluconazole and/or echinocandins. *MSH2* gene sequence of wild-type revealed the presence of mutations in isolates CG1 and CG3 [V239L], CG4 [E459K], and CG5 [A313V]; the sequence of the *MSH2* gene did not change after 10 days of propagation in the presence of micafungin. Before the diagnosis of candidemia, two out of the five patients received fluconazole or micafungin; all of them received an echinocandin ($n = 4$) or voriconazole ($n = 1$) after the diagnosis. As none of the patients yielded isolates showing secondary resistance to echinocandins, the role of the *MSH2* gene mutations is unknown.

We recently reported that this set of five wild-type isolates developed phenotypic resistance to echinocandins after *in vitro* exposure to increasing concentrations of micafungin.[5] Here, we report that the isolates were also able to acquire resistance to echinocandins after exposure to a low and constant concentration of micafungin. Anatomical sites such as the abdomen or colonized mucosa where concentrations of echinocandins are purportedly low, could act as reservoirs for echinocandin-resistant *C. glabrata* isolates.[3,12,13] These observations suggest that spontaneous mutations in *C. glabrata* isolates might be selected

Table 1. Susceptibility of *Candida glabrata* (CG) isolates to micafungin and anidulafungin during exposure to low micafungin concentrations on agar plates.

Isolates	MICs (mg/l) obtained by EUCAST and *FKS2* mutations									
	CG1		CG2		CG3		CG4		CG5	
	MYC/AND	*FKS2*	MYC/AND	*FKS2*	MYC/AND	*FKS2*	MYC/AND	*FKS2*	MYC/AND	*FKS2*
$MIC_{Initial}$	≤0.015/0.031	WT	≤0.015/≤0.015	WT	≤0.015/≤0.015	WT	≤0.015/≤0.015	WT	≤0.015/≤0.015	WT
MIC (plate-to-plate propagation)										
1st day	≤0.015/0.031	WT	≤0.015/≤0.015	WT	≤0.015/≤0.015	WT	≤0.015/≤0.015	WT	≤0.015/≤0.015	WT
2nd day	**2/2**	**S663P**	≤0.015/≤0.015	WT	≤0.015/≤0.015	WT	≤0.015/≤0.015	WT	**2/1**	**S663P**
3rd day	**2/2**	**S663P**	0.031/0.031	WT	**1/2**	**S663P**	**2/1**	**delF658**	**2/2**	**S663P**
4th day	**2/2**	**S663P**	**2/2**	**W715L**	**2/4**	**S663P**	**2/1**	**delF658**	**2/2**	**S663P**
5th day	**4/2**	**S663P**	**2/2**	**W715L**	**2/4**	**S663P**	**2/1**	**delF658**	**2/2**	**S663P**
6th day	**2/2**	**S663P**	**2/2**	**W715L**	**4/4**	**S663P**	**2/1**	**delF658**	**2/2**	**S663P**
7th day	**2/2**	**S663P**	**2/2**	**W715L**	**4/4**	**S663P**	**2/1**	**delF658**	**2/2**	**S663P**
8th day	**2/2**	**S663P**	**2/2**	**W715L**	**4/4**	**S663P**	**2/1**	**delF658**	**2/2**	**S663P**
9th day	**2/2**	**S663P**	**2/2**	**W715L**	**2/4**	**S663P**	**2/1**	**delF658**	**4/2**	**S663P**
10th day (MIC_{final})	**2/2**	**S663P**	**2/2**	**W715L**	**4/4**	**S663P**	**2/1**	**delF658**	**4/2**	**S663P**

No mutations in *FKS1* gene were found.
AND, anidulafungin; MYC, micafungin.
Bold characters represent phenotypic resistance and *FKS2* mutation.

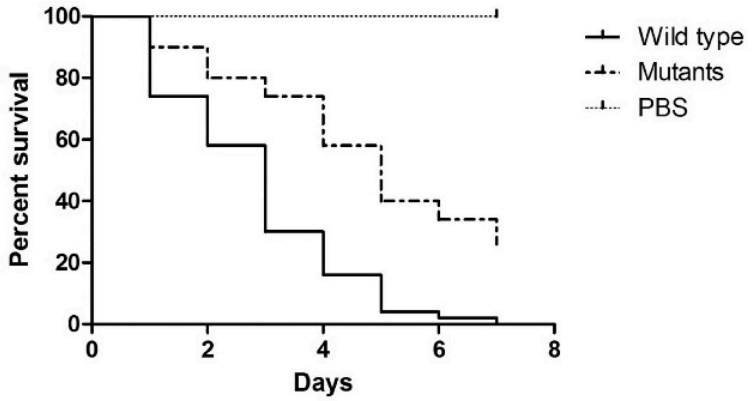

Figure 1. Survival curves of *Galleria mellonella* larvae following inoculation with 5×10^6 cfu per larva of wild-type isolates and their corresponding mutant isolates, and the larvae injected with sterile PBS as control.

during prolonged exposure to low micafungin concentrations owing to the high susceptibility of the wild-type population to this drug. However, the kind of mutation acquired in the HS1 region under these conditions is not always predictable. Compared with the previous study only two isolates (CG1 and CG4) showed the same amino acid substitutions in both experiments.

The apparently high potential of *C. glabrata* to acquire *in vitro* echinocandin resistance seems contradictory with respect to the low reported rate of resistance in invasive clinical isolates possibly because of the lower fitness of mutant isolates than of wild-type isolates.[1,14] We did not find differences in kinetic parameters between the wild-type isolates and the mutant isolates, respectively, in terms of the average growth rate (5.12×10^{-6} s^{-1} and 5.27×10^{-6} s^{-1}) and time to maximum rate (1.1×10^5 s and 7.5×10^4 s). However, we found differences ($P < .0001$) in the median survival of larvae infected with mutant isolates (5 days) and wild-type isolates (3 days) (Fig. 1). The median survival of the wild-type and the corresponding mutant isolate, respectively, was not homogeneous: CG1, 2.5 vs. 5 days; CG2, 5 vs. 4.5 days; CG3, 2.5 vs. 4 days; CG4, 3 vs. 5 days; and CG5, 2.5 vs. 4.5 days. Interestingly, the isolate causing lower mortality in the *Galleria* model was

that showing the previously unreported mutation (W715L). Previous studies have reported an association between genome plasticity and antifungal drug susceptibility in *C. glabrata*.[11,15] Although we only studied 5 isolates, there might be an association between the kind of mutation and fitness, however, contradictory results have been reported in *C. glabrata*.[10,16,17]

Our study is subjected to limitations. First, we only studied five isolates and the differences among isolates in terms of virulence may not reach statistical significance if more isolates were studied. Second, *G. mellonella* is a cheap and easy to perform model suitable for screening compared to vertebrate models, but may not mirror the human host. Finally, Vallabhaneni and colleagues reported *FKS* mutations in *C. glabrata* from patients who never received any echinocandin.[18] Therefore, the presence echinocandin resistance does not necessarily impair the invasiveness of the isolates.

We conclude that echinocandin resistance is relatively easy to achieve when *C. glabrata* clinical isolates are exposed to low and constant concentrations of micafungin. The impact of the *FKS* mutations on the fitness of *C. glabrata* isolates needs future studies.

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Declaration of interest

J.G. has received educational grants from Astellas, Gilead, MSD, Scynexis, and United Medical; he has also received funds for research from Fondo de Investigación Sanitaria, Gilead, Scynexis, and Cidara. R.C. has received funds for speaking at symposia organized on behalf of Gilead, and MSD. P.E. has received funds for research from Fondo de Investigación Sanitaria.

All other authors report no conflicts of interest. The autores alone are responsible for the content and the writing of the paper.

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6.1.3. Artículo 3: Mutant prevention concentration and mutant selection window of micafungin and anidulafungin in clinical *Candida glabrata* isolates.

En este estudio se calculó la concentración preventiva de mutantes (MPC), la ventana de selección de mutantes (MSW), y la frecuencia de mutación a micafungina y anidulafungina en cepas clínicas de *C. glabrata* productoras de candidemia. Adicionalmente, se estudió la cinética de crecimiento y la virulencia entre cepas clínicas sensibles y sus respectivos aislados resistentes, así como la secuencia del gen *MSH2* con la exposición a antifúngicos previos en los pacientes y su pronóstico.

Los inóculos ajustados de 20 aislados de *C. glabrata* susceptibles a las equinocandinas e intermedios a fluconazol se cultivaron directamente sobre placas de agar con diferentes concentraciones de equinocandinas, e inspeccionaron diariamente hasta el 5º día de incubación. Se determinó la sensibilidad antifúngica por EUCAST a las colonias individuales obtenidas, y en aquellas fenotípicamente resistentes se secuenciaron los genes *FKS*. Se compararon las cepas resistentes y sensibles en términos de formación de biopelículas, parámetros cinéticos de crecimiento, virulencia (mediante el modelo de *Galleria mellonella*), y alteraciones en el gen *MSH2*.

Micafungina y anidulafungina mostraron similares MPCs (0,06 - 2 mg/L y 0,25 - 2 mg/L, respectivamente), MSW (0,015 - 2 mg/L, para ambas equinocandinas) y frecuencia de mutación (3,7 $x10^{-8}$ y 2,8 $x10^{-8}$, respectivamente). La placa con concentración de 1 mg/L (mayor concentración que permitió el crecimiento) dio lugar a la proliferación de un total de 32 colonias fenotípicamente resistentes procedentes de 12 aislados. Solo en 21 de las 32 colonias se observaron mutaciones en el gen *FKS2*. No se encontraron diferencias en la formación de biopelículas, en los parámetros cinéticos estudiados, ni en la virulencia entre aislados de tipo salvaje o mutante. Las mutaciones en el gen *MSH2* no se relacionaron con las características clínicas estudiadas.

Se concluyó que *C. glabrata* fue capaz de desarrollar resistencia secundaria *in vitro* en concentraciones de equinocandinas <2 mg/L. La adquisición de resistencia secundaria no se relacionó con la patogenicidad, ni con la presencia de mutaciones en el gen *MSH2*.

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Mutant Prevention Concentration and Mutant Selection Window of Micafungin and Anidulafungin in Clinical *Candida glabrata* Isolates

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ABSTRACT We report the mutant prevention concentration (MPC) and mutant selection window (MSW) for micafungin and anidulafungin administered to treat *Candida glabrata*. We also determine the mutation frequency. We studied 20 echinocandin-susceptible, fluconazole-intermediate, and *FKS* wild-type *C. glabrata* isolates. Adjusted inocula were stroked directly onto Sabouraud agar plates containing different concentrations of micafungin or anidulafungin and visually inspected daily for up to 5 days of incubation. Individual colonies growing on the plates containing echinocandins at 1 mg/liter were selected for antifungal susceptibility testing. The *FKS* genes of the resulting individual phenotypically resistant colonies were sequenced, and the MPC, MSW, and mutation frequency were determined. Biofilm was quantified, and the growth kinetics and virulence (*Galleria mellonella* model) of the resulting individual *FKS* mutant colonies were studied. For micafungin and anidulafungin, we found similar results for the MPC (0.06 to 2 mg/liter and 0.25 to 2 mg/liter, respectively), MSW (0.015 to 2 mg/liter for both echinocandins), and mutation frequency (3.7×10^{-8} and 2.8×10^{-8}, respectively). A total of 12 isolates were able to grow at 1 mg/liter on echinocandin-containing plates, yielding a total of 32 phenotypically resistant colonies; however, *FKS2* mutations (ΔF658, S663P, W715L, and E655A) were observed only in 21 colonies. We did not find differences in biofilm formation, the kinetic parameters studied, or the median survival of larvae infected by wild-type isolates and the resulting individual *FKS2* mutant colonies. Echinocandin concentrations lower than 2 mg/liter can lead to selection of resistance mutations in *C. glabrata* isolates *in vitro*.

KEYWORDS *Candida glabrata*, echinocandins, MPC, MSW, *FKS* mutation, *Galleria mellonella*

Candida glabrata is one of the most clinically relevant causes of candidemia, and the incidence of candidemia caused by this entity seems to be on the rise (1–3). Echinocandins are currently recommended as the first-line treatment for invasive candidiasis (4–6), and resistance may complicate the management of patients infected by *C. glabrata*.

Resistance mutations have been identified in patients receiving long-term treatment with echinocandins and *in vitro* after exposure to increasing or constantly low concen-

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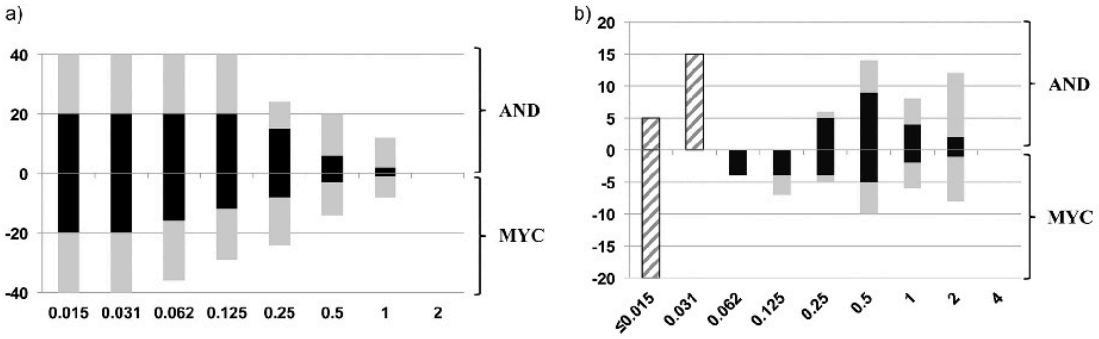

FIG 1 (a) Number of original isolates growing on anidulafungin-containing plates (positive *y* axis) and on micafungin-containing plates (negative *y* axis) after 24 h (black bars) and 5 days (gray bars) of incubation. (b) Distribution of MICs (striped bars) and MPCs (solid bars) of anidulafungin (positive *y* axis) and micafungin (negative *y* axis) after 24 h (black bars) and 5 days of incubation (gray bars).

trations of echinocandins (7, 8). Echinocandin resistance is associated with the presence of mutations in hot-spot regions of the genes *FKS1* and *FKS2* (3, 9, 10).

The cause of increased resistance to echinocandins is unclear. However, 2 recent studies showed that the abdominal compartment and/or colonized mucosa of patients with invasive candidiasis could act as a hidden reservoir of echinocandin-resistant *C. glabrata* isolates (11, 12). A potential explanation for this finding is the selection of resistance mutations in peritoneal fluid in the presence of low echinocandin concentrations, which are insufficient to inhibit selection of mutations but might promote the selection of resistant mutants (13). In addition, the *MSH2* mutator phenotype or specific genotypes may contribute to the development of resistance to echinocandins (14, 15).

The parameters mutant prevention concentration (MPC) and mutant selection window (MSW) are useful when attempting to optimize antibacterial treatment, minimize the emergence of resistant mutants, and understand treatment failure (16). These parameters are mostly unknown for *Candida* spp., although they might be particularly relevant for *C. glabrata*.

We report the micafungin and anidulafungin MPC and MSW in *C. glabrata* and the corresponding mutation frequency. Furthermore, we determined whether the acquisition of *FKS* mutations entailed a cost for the isolates in terms of fitness and virulence.

(This study was partially presented at the 27th European Congress of Clinical Microbiology and Infectious Diseases in Vienna, Austria, 2017 [ePoster no. EP0626].)

RESULTS

Echinocandin MPC and MSW and mutation frequency. All of the studied isolates were echinocandin susceptible, fluconazole intermediate, and *FKS1* and *FKS2* wild type. The number of isolates growing on plates containing micafungin or anidulafungin increased when the incubation period was prolonged to 5 days; overall, the number of isolates growing on anidulafungin-containing plates was higher than the number growing on micafungin-containing plates (Fig. 1a).

The MIC, MPC, and MSW values for each isolate and overall are shown in Table 1. The observed ranges of micafungin and anidulafungin MPCs were wide (Fig. 1b); none of the isolates was able to grow at echinocandin concentrations above 1 mg/liter. The geometric mean MPCs obtained after 24 h of incubation were significantly lower than the MPCs obtained after 5 days, regardless of the drug studied ($P = 0.001$). However, the geometric mean MPC of anidulafungin was significantly higher than that of micafungin after 24 h of incubation (0.55 mg/liter versus 0.25 mg/liter; $P = 0.046$), although the differences did not reach statistical significance after 5 days of incubation (1.15 mg/liter versus 0.73 mg/liter) (Fig. 1b and Table 1). Nevertheless, the MSW of anidulafungin and micafungin was identical after 24 h and 5 days of incubation of the plates (Fig. 1b and Table 1).

TABLE 1 Micafungin and anidulafungin MIC, MPC, and MSW after 24 h and 5 days of incubation for each isolate

Isolate	Value(s) (mg/liter) for[a]:					
	MYC			AND		
	MIC	MPC	MSW	MIC	MPC	MSW
1	**0.015**	**2/2**	**0.015–2/0.015–2**	**0.03**	**0.5/2**	**0.03–0.5/0.03–2**
2	**0.015**	**0.25/2**	**0.015–0.25/0.015–2**	**0.015**	**1/2**	**0.015–1/0.015–2**
3	**0.015**	**0.125/2**	**0.015–0.125/0.015–2**	**0.015**	**0.5/2**	**0.015–0.5/0.015–2**
4	**0.015**	**0.5/2**	**0.015–0.5/0.015–2**	**0.03**	**0.5/2**	**0.03–0.5/0.03–2**
5	**0.015**	**0.5/0.5**	**0.015–0.5/0.015–0.5**	**0.03**	**0.25/2**	**0.03–0.25/0.03–2**
6	**0.015**	**0.25/2**	**0.015–0.25/0.015–2**	**0.03**	**0.5/1**	**0.03–0.5/0.03–1**
7	**0.015**	**1/2**	**0.015–1/0.015–2**	**0.03**	**0.5/1**	**0.03–0.5/0.03–1**
8	**0.015**	**0.125/1**	**0.015–0.125/0.015–1**	**0.03**	**0.5/2**	**0.03–0.5/0.03–2**
9	**0.015**	**0.25/1**	**0.015–0.25/0.015–1**	**0.03**	**0.25/2**	**0.03–0.25/0.03–2**
10	**0.015**	**0.25/0.5**	**0.015–0.25/0.015–0.5**	**0.03**	**1/2**	**0.03–1/0.03–2**
11	**0.015**	**0.06/0.5**	**0.015–0.06/0.015–0.5**	**0.03**	**2/2**	**0.03–2/0.03–2**
12	**0.015**	**1/2**	**0.015–1/0.015–2**	**0.015**	**2/2**	**0.015–2/0.015–2**
13	0.015	0.125/0.125	0.015–0.125/0.015–0.125	0.03	0.5/0.5	0.03–0.5/0.03–0.5
14	0.015	0.06/0.125	0.015–0.06/0.015–0.125	0.015	1/1	0.015–1/0.015–1
15	0.015	0.125/0.125	0.015–0.125/0.015–0.125	0.03	0.5/0.5	0.03–0.5/0.03–0.5
16	0.015	0.06/0.25	0.015–0.06/0.015–0.25	0.03	0.25/0.25	0.03–0.25/0.03–0.25
17	0.015	0.5/1	0.015–0.5/0.015–1	0.015	0.25/0.5	0.015–0.25/0.015–0.5
18	0.015	0.06/1	0.015–0.06/0.015–1	0.03	0.25/0.5	0.03–0.25/0.03–0.5
19	0.015	0.5/0.5	0.015–0.5/0.015–0.5	0.03	0.5/1	0.03–0.5/0.03–1
20	0.015	0.5/0.5	0.015–0.5/0.015–0.5	0.03	1/1	0.03–1/0.03–1
Overall (GM)	0.015	0.25/0.73		0.025	0.55/1.16	
Range	0.015	0.06–2/0.125–2	0.015–2/0.015–2	0.015–0.03	0.25–2/0.25–2	0.015–2/0.015–2

[a]MYC, micafungin; AND, anidulafungin. Values for MPC and MSW are shown for 24 h/5 days of incubation for each isolate. Boldface characters (isolates codes 1 to 12) represent isolates that were able to grow on plates containing 1 mg/liter of echinocandin.

A total of 12 isolates (numbered 1 to 12; Table 1) grew on agar plates containing 1 mg/liter of echinocandin and yielded 32 colonies, 12 in the micafungin-containing plates and 20 in the anidulafungin-containing plates (Fig. 2). These colonies proved to be phenotypically resistant to both echinocandins according to the EUCAST procedure (subculture on an antifungal-free plate after 24 h of incubation) and despite the fact that only 21 carried *FKS2* mutations, whereas the *FKS1* sequence was wild type (11 in the micafungin-containing plates and 10 in the anidulafungin-containing plates) (Table 2 and Fig. 2). The mutations found were ΔF658 (n = 14), S663P (n = 4), W715L (n = 2), and E655A (n = 1) (Fig. 2). Significant differences were found in the echinocandin geometric mean MICs against the 11 *FKS2* wild-type isolates and the 21 *FKS2* mutant colonies (P = 0.02) (Table 2 and Fig. 2). However, the geometric mean MICs of fluconazole against both groups of isolates did not differ significantly (6.73 mg/liter

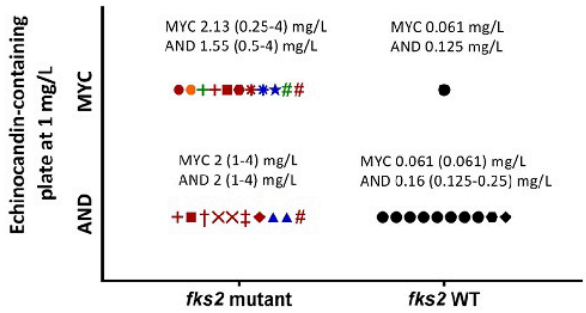

FIG 2 Echinocandin-resistant colonies growing on echinocandin-containing plates (at 1 mg/liter) grouped according to the presence or absence of *FKS2* mutations. The range and geometric mean echinocandin MICs for each group are shown. Each symbol represents a colony; colonies with the same symbol shape come from the same isolate. The colors represent different *FKS2* mutations (red, ΔF658; Blue, S663P; Green, W715L; orange, E655A). Symbols in black represent echinocandin-resistant but *FKS2* wild-type colonies.

TABLE 2 Study of virulence in the *Galleria mellonella* larvae[a]

Isolate no. and echinocandin plate exposure	MIC (mg/liter)		*FKS2* HS1 sequence (no. of colonies)	Median survival (days)
	MYC	AND		
1				
None	0.015	0.03	WT	5
MYC	4	2	ΔF658 (1)	6
	0.25	0.5	E655A (1)	5
	0.06	0.125	WT (1)	5.5
AND	0.06	0.125	WT (6)	4.5
	0.06	0.25	WT (2)	4
2				
None	0.015	0.015	WT	5
MYC	0.5	0.5	W715L (1)	5
	2	1	ΔF658 (1)	5
AND	2	2	ΔF658 (1)	5.5
3				
None	0.015	0.015	WT	3
MYC	4	4	ΔF658 (1)	3
AND	2	4	ΔF658 (1)	3
4				
None	0.015	0.03	WT	3.5
MYC	4	2	ΔF658 (1)	3.5
AND	0.06	0.25	WT (1)	4.5
5				
None	0.015	0.03	WT	2
AND	1	1	ΔF658 (1)	2
6				
None	0.015	0.03	WT	3
MYC	4	2	ΔF658 (1)	5.5
	4	2	S663P (1)	3.5
7				
None	0.015	0.03	WT	2
MYC	2	1	S663P (1)	2
8				
None	0.015	0.03	WT	6
AND	4	2	ΔF658 (1)	6.5
	2	2	ΔF658 (1)	6
9				
None	0.015	0.03	WT	2.5
AND	1	2	ΔF658 (1)	2.5
10				
None	0.015	0.03	WT	4
AND	1	2	ΔF658 (1)	7
	0.06	0.25	WT (1)	4
11				
None	0.015	0.03	WT	3
AND	2	2	S663P (1)	3
	4	2	S663P (1)	4
12				
None	0.015	0.015	WT	3
MYC	4	2	W715L (1)	3
	2	4	ΔF658 (1)	3.5
AND	4	2	ΔF658 (1)	5

[a]Results are larvae infected by 32 colonies (*FKS2* mutant and *FKS2* wild type) obtained from the 12 isolates streaked onto plates containing 1 mg/liter echinocandin.

versus 5.75 mg/liter). *FKS2* mutations and phenotypic resistance were stable after 5 propagations on echinocandin-free agar plates. Genotyping showed no potential contamination of the isolates during the study (data not shown).

The geometric mean of the mutation frequency and its range in the presence of micafungin (3.7×10^{-8} and 6.4×10^{-8} to 2.3×10^{-8}) did not differ from those of anidulafungin (2.8×10^{-8} and 1.5×10^{-7} to 1.7×10^{-8}) ($P = 0.5$). However, if only colonies with *FKS2* mutations are selected, the geometric mean of the mutation frequency and its range were lower and significantly different ($P = 0.02$) in the presence of micafungin (3.4×10^{-8} and 5.3×10^{-8} to 2.2×10^{-8}) than that in the presence of anidulafungin (2.2×10^{-8} and 3.9×10^{-8} to 1.7×10^{-8}).

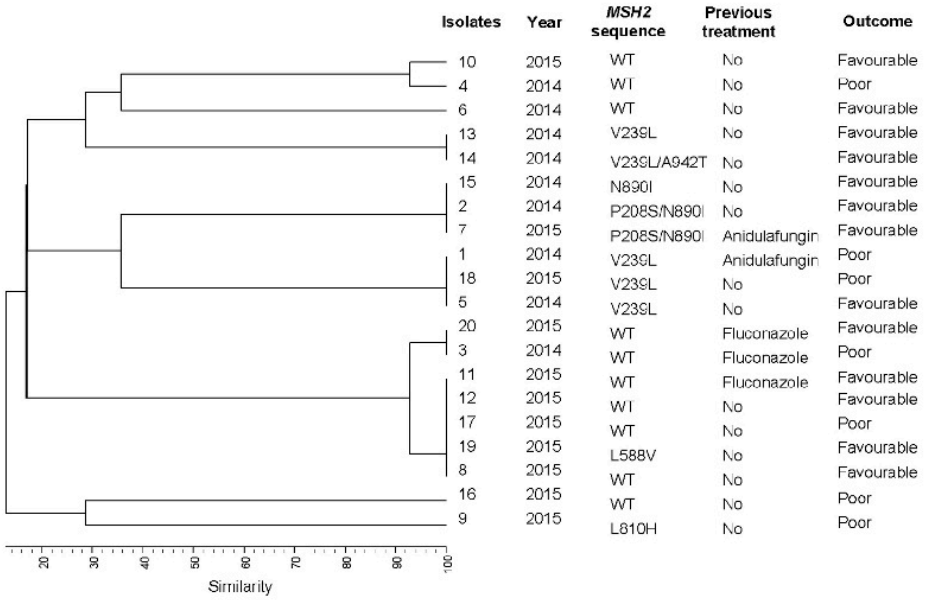

FIG 3 Genetic relationship between the 20 *Candida glabrata* isolates studied; the *MSH2* sequence of each isolate, patient outcome (mortality), and the previous antifungal received are also shown.

Genotyping analysis and *MSH2* gene sequencing. Genotyping of the 20 wild-type isolates revealed the presence of 10 genotypes, 5 of which were clusters involving 75% of isolates (Fig. 3). The *MSH2* gene sequence of wild-type isolates revealed the presence of the following mutations in 50% of isolates (Fig. 3): V239L ($n = 4$), V239L/A942T ($n = 1$), P208S/N890I ($n = 2$), N890I ($n = 1$), L810H ($n = 1$), and L588V ($n = 1$). L810H and L588V have not been previously described. Most of the *MSH2* mutations were found in isolates involved in clusters, and although the bulk of isolates from each cluster showed the same mutation, some isolates showed an *MSH2* sequence that differed from the remaining isolates in the cluster. The presence of *MSH2* mutations in wild-type isolates was not associated with the secondary acquisition of resistance to echinocandins, type of *FKS2* mutation, patient outcome, or previous antifungal treatment.

Pathogenicity study of wild-type and resulting *C. glabrata* echinocandin-resistant isolates. We did not find differences in the average growth/time to maximum rate between the wild-type isolates (4.76×10^{-6} s^{-1}/8.36×10^{4} s) and the *FKS2* mutant colonies (4.69×10^{-6} s^{-1}/1.16×10^{5} s).

Wild-type isolates were classified as low (65%) or moderate (35%) biofilm formers. They exhibited high (80%) and moderate (20%) metabolic activity. The individual *FKS2* mutant colonies were low (81%) or moderate (19%) biofilm formers and exhibited high (33.3%) and moderate (66.7%) metabolic activity. The differences did not reach statistical significance.

We found no differences in the median survival of larvae infected with *FKS2* mutant colonies (4 days) and those infected by the wild-type isolates (4 days), regardless of the type of *FKS2* mutation or the echinocandin-containing plate with the mutant (Table 2 and Fig. 4). All *FKS2* mutant colonies in the same wild-type isolate showed similar median survival regardless of the type of *FKS2* mutations found; however, we observed median differences in survival between isolates with the same *FKS2* mutation.

DISCUSSION

Our study shows that *C. glabrata* isolates are able to develop secondary resistance to echinocandins when the drug concentration is below 2 mg/liter. The presence of mutations in the *MSH2* gene did not seem to influence the selection of resistance

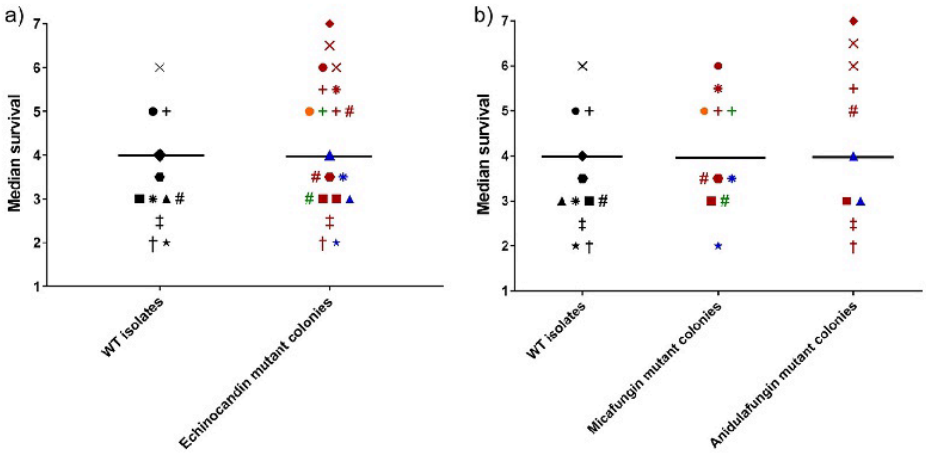

FIG 4 (a) Median survival of wild-type (WT) isolates and mutant colonies on plates containing 1 mg/liter echinocandin and the median survival of each group. (b) Median survival of WT isolates and mutant colonies on plates containing 1 mg/liter micafungin and anidulafungin and the median of each group. Phenotypic echinocandin-resistant isolates showing the WT *FKS2* gene were excluded from the analysis. Each symbol represents a colony; colonies with the same symbol shape come from the same isolate.

mutations, and *FKS2* mutations did not have a significant impact on the mortality of *G. mellonella*-infected larvae.

The rate of echinocandin resistance in *C. glabrata* has been increasing over the last few years, particularly in areas of northern Europe and North America (2, 3). However, the rate of resistance to echinocandins in *C. glabrata* in other regions, such as Spain, is much lower (1, 17). The reasons for these apparently heterogeneous findings are unclear, although they could be the result of variations in policies toward the use of echinocandins. It has been shown that long-term treatment with echinocandins is a risk factor for the presence of *C. glabrata*-resistant isolates (2, 18, 19).

Even though few patients are primarily infected by echinocandin-resistant isolates, promotion of *in vitro* resistance is relatively easy to achieve in *C. glabrata* (7, 8, 20–23). We recently showed that *C. glabrata* isolates could become echinocandin resistant *in vitro* after serial propagation onto plates containing low to increasing micafungin concentrations (7, 8). Consequently, the combination of a high inoculum and low concentrations of drug within the MSW are required to obtain resistant isolates. The present study was designed to complement our previous observations by assessing the mutation frequency, the MPC, and the MSW. Ideally, the concentration of an antimicrobial drug in the body should be above the MPC to rule out the selection of resistant mutants (i.e., *FKS* mutants), particularly at sites where *C. glabrata* cells are abundant. The parameters MPC and MSW were previously used for antibacterial drugs to optimize the response to antimicrobial treatment and to optimize prevention of emergence of resistant isolates (24–27). However, they have received little attention in the study of *Candida* species infection.

We found that concentrations of micafungin and anidulafungin of ≥2 mg/liter could prevent the emergence of *C. glabrata*-resistant mutants. Furthermore, concentrations between the MIC and 1 mg/liter may enable the emergence of these mutants. This observation is consistent with findings from our previous study, in which a concentration of 0.031 mg/liter of micafungin, which is within the MSW, was able to promote the presence of *C. glabrata*-resistant mutants (8). The echinocandin MIC endpoint is defined as a ≥50% reduction in fungal growth in the presence of the drug compared with the drug-free control well, meaning that a residual number of viable yeast cells may be present. However, it is not surprising that the EUCAST procedure could prove inefficient for selection of mutants, considering the inoculum suspension plated (1×10^5 CFU/ml) and the *C. glabrata* mutation frequency in the presence of micafungin or anidulafungin

reported here (5×10^{-8} to 2×10^{-8} and 4×10^{-8} to 2×10^{-8}, respectively). For that reason, and as reported for bacteria, we used very high inocula (10^9 CFU/ml) to ensure the presence of mutants in order to calculate the MPC and the MSW.

MPCs should be evaluated in line with drug pharmacokinetics and the suspected origin of the infection, because using high doses to overcome the MPC in tissues may induce drug-related toxicity. This limitation can be addressed by combining various drugs, as previously shown for antibacterials (27). However, considering the poor support that antifungal combination has received in recent guidelines (5, 6), this approach may be reserved for scenarios of high echinocandin resistance rates. Pharmacokinetic studies reported the maximum concentration of drug in serum (C_{max}) of micafungin, anidulafungin, and caspofungin to be equal to 4.95 mg/liter, 3.5 mg/liter, and 7.64 mg/liter, respectively, after standard single doses (28, 29). However, in patients with peritonitis, the administration of a dose of micafungin yielded consistently low levels of the drug in the peritoneum (below 2 mg/liter), thus enabling the emergence of mutations in *C. glabrata* over time (13). These concentrations of echinocandins in the bloodstream clearly exceed the MPC and thus may be able to prevent the selection of resistance mutations because of the purportedly low number of circulating cells. However, echinocandins are partially excreted in feces, where the number of *C. glabrata* cells may be much higher, enabling spontaneously generated mutants to become selected (30). The presence of *FKS* mutants in *C. glabrata* isolates from sites with impaired penetration of candins, such as the skin or the peritoneum, has been reported (11–13).

According to EUCAST, *C. glabrata* isolates with micafungin or anidulafungin MICs above 0.031 mg/liter and 0.062 mg/liter, respectively, are resistant (31, 32). Of the 32 phenotypically resistant colonies found, 11 did not harbor *FKS* mutations. This phenomenon was more frequently observed in colonies grown on plates containing anidulafungin than on plates containing micafungin, and the MICs tended to be no more than 1-fold or 2-fold concentrations above the breakpoint. To date, the *FKS* mutation is the only reported mechanism of resistance, and *FKS* wild-type isolates with slightly high echinocandin MICs might not be considered truly resistant. Other authors have reported the same observations (particularly on plates containing anidulafungin), even in isolates with higher echinocandin MICs, thus warranting future research into this issue (21, 33). In order to calculate the mutation frequency, we chose 1 mg/liter of echinocandins as our minimum, as colony counting was not possible at lower concentrations. We did not find significant differences between micafungin and anidulafungin if the mutation frequency was calculated based on resistant colonies. However, taking into account only the mutants, significant differences were observed, and the mutation frequency reported here is similar to that of other studies (21, 23).

The apparent ability of *C. glabrata* isolates to acquire resistance to echinocandins *in vitro* is not consistent with the low number of resistant isolates from clinical invasive samples. A potential explanation is that the fitness of mutant cells is inferior to that of the wild type. We recently reported a positive correlation in mortality between the patients and the *G. mellonella* model, showing that this model can be suitable for the study of virulence in *Candida* (34). Overall, we did not find significant differences in median survival between wild-type isolates and their *FKS2* mutant colonies. However, the wild-type isolates were more virulent than the resulting *FKS2* mutant colonies in 5 of the 12 isolates, whereas median survival was similar to that of the wild-type isolates in the remaining mutant colonies. These observations, which are not consistent with our recently reported results, may be explained in part by the differences between the mean mortality of larvae infected by wild-type isolates versus those infected by *FKS* mutant isolates reported previously (3 versus 5 days) (8) or in the present study (4 versus 4 days). Similarly, we found no differences in biofilm formation between mutant and wild-type isolates. *C. glabrata* frequently forms biofilms featuring low biomass and high metabolic activity (35). Therefore, the lack of differences in biofilm formation between mutant and wild-type isolates is not surprising. Biofilm formation is lower in *C. glabrata* than in *C. albicans*, and the patients infected by the latter have higher

mortality (36). The low early mortality (within 30 days of diagnosis) of our patients also may be associated with the low biofilm formation.

Although some authors showed that the *MSH2* mutator phenotype or specific genotypes may contribute to the development of resistance to echinocandins (14, 15), we were unable to confirm these findings, as the modifications in the *MSH2* gene were not related to more marked acquisition of echinocandin resistance *in vitro*.

Alternative mechanisms of echinocandin resistance, such as compensatory mutations or genomic rearrangement, have been reported in *C. glabrata* (15, 37). However, we cannot rule out the presence of the heteroresistant subpopulations rather than genetic drift in this clonal species (38).

We conclude that *C. glabrata* isolates are able to develop secondary resistance to echinocandins when the concentrations of the drugs are below 2 mg/liter. The presence of mutations in the *MSH2* gene did not seem to influence the promotion of resistance, and *FKS2* mutations did not have a significant impact on the mortality of *G. mellonella*-infected larva.

MATERIALS AND METHODS

Yeast isolates and patients. We studied 20 *Candida glabrata* isolates recovered from blood cultures of patients with candidemia (1 isolate per patient) admitted to Gregorio Marañón Hospital (Madrid, Spain) between 2014 and 2015. The isolates were identified using chromogenic agar plates and confirmed by amplification and sequencing of the ITS1-5.8S-ITS2 regions (39). A total of 25% of the patients studied had received fluconazole or anidulafungin during the month before the diagnosis of candidemia. Early mortality (within 30 days of diagnosis) and late mortality (30 days after diagnosis) were 25% and 35%, respectively.

Antifungal susceptibility testing. All isolates were tested for susceptibility to micafungin (Astellas Pharma, Inc., Tokyo, Japan), anidulafungin, and fluconazole (Pfizer Pharmaceutical Group, New York, NY, USA) according to the EUCAST EDef 7.2 microdilution procedure (31, 32, 40, 41). The echinocandins and fluconazole were tested at concentrations ranging from 0.015 to 8 mg/liter and 0.25 to 128 mg/liter, respectively. Inoculated plates were incubated for 24 h at 35°C. Strains were classified according to the resistance breakpoints proposed by EUCAST: micafungin, MIC of >0.031 mg/liter; anidulafungin, MIC of >0.062 mg/liter; and fluconazole, MIC of >32 mg/liter (31, 32, 40).

Echinocandin mutant prevention concentration and mutant selection window. Exposure to echinocandins on agar plates was as previously described, with some modifications (7). Briefly, isolate suspensions were adjusted to 3×10^9 to 7×10^9 CFU/ml (mean of $4.8 \times 10^9 \pm 0.96 \times 10^9$) using a Neubauer chamber and stroked directly (100 μl) onto Sabouraud agar plates containing 8 concentrations (0.015 mg/liter to 2 mg/liter; 2-fold concentrations) of micafungin and anidulafungin. Plates were incubated at 35°C and visually inspected every 24 h for up to 5 days of incubation. MPCs were defined as the lowest echinocandin concentration leading to complete inhibition of fungal growth on echinocandin-containing agar plates at 2 time points (24 h and 5 days) (16).

The MPCs obtained at the 2 incubation time points were compared using the Wilcoxon signed-rank test (IBM SPSS Statistics for Windows, version 21.0; Armonk, NY, USA). A P value of <0.05 was considered statistically significant.

The MSW for each isolate was defined as the range of concentrations between the MIC, obtained by microdilution, and the MPC, considering the 2 incubation time points (24 h and 5 days) (42).

Mutation frequency. The calculation of mutation frequency was based on an echinocandin concentration of 1 mg/liter; lower concentrations may not have enabled individualization of colonies owing to heavy growth. The individual colonies growing on the plates containing echinocandins at concentrations equal to 1 mg/liter after 5 days of incubation were studied for susceptibility to micafungin, anidulafungin, and fluconazole according to the EUCAST procedure, and the hot spots of *FKS1* and *FKS2* genes were sequenced (43, 44). Mutation frequency was defined as the ratio between the number of phenotypically resistant colonies on micafungin-containing or anidulafungin-containing plates and the number of cells stroked. The mutation frequencies obtained for both echinocandins were compared (Wilcoxon signed-rank test; IBM SPSS Statistics for Windows, version 21.0).

***MSH2* sequencing.** The *MSH2* gene of the wild-type and resulting *C. glabrata* echinocandin-resistant isolates was amplified and sequenced according to Delliere et al. (14), with the following modifications: 1.25 U of AmpliTaq gold (Applied Biosystems), 0.2 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, and 100 ng of extracted DNA.

Microsatellite typing. The wild-type and the resulting *C. glabrata* echinocandin-resistant isolates were genotyped using a panel of 14 microsatellite markers (45–48). Singleton genotypes were defined as those found only once, whereas a cluster was defined as the presence of ≥2 isolates with the same genotype.

Pathogenicity study of wild-type and resulting *C. glabrata* echinocandin-resistant isolates. The *in vitro* growth kinetics, biofilm quantification, and virulence on final-instar larvae of *Galleria mellonella* of the wild-type isolates and the resulting individual *FKS* mutant colonies were studied as previously described (8).

The average growth rate and time to maximum rate were calculated (49). Briefly, 100 μl of adjusted inocula (3×10^5 CFU/ml) of each isolate was added to 100 μl of double-concentrated RPMI 1640 medium supplemented with 2% glucose (Merck KGaA, Darmstadt, Germany) and morpholinepropanesulfonic acid (MOPS; Sigma-Aldrich, Co., St. Louis, MO, USA) in flat-bottomed 96-well microdilution trays. Each study was performed in triplicate. Trays were incubated at 35°C with moderate shaking in a spectrophotometer (Thermo Fisher Scientific, Madrid, Spain) for 35 h. The optical density in each well was measured every 15 min at 490 nm to calculate the kinetic parameters (average growth rate and time to maximum rate). Differences between kinetic parameters were studied using the Kruskal-Wallis test.

The biofilm was formed according to the method proposed by Marcos-Zambrano and colleagues (35). Briefly, the cells were grown at 30°C with shaking overnight on 10 ml of yeast-peptone-dextrose broth (Difco, Becton Dickinson, Madrid, Spain) before being washed and resuspended in 5 ml of RPMI 1640 broth medium adjusted to approximately 1×10^6 cells/ml. A total of 100 μl of the suspension was inoculated in 96-well trays and incubated for 24 h at 37°C. Each strain was tested in triplicate. Biofilm production was quantified using crystal violet staining; the metabolic activity of biofilm was measured using the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt] reduction assay. We followed the cutoff points proposed in a previous paper to classify the isolates as low, moderate, and high biofilm forming and as having low, moderate, and high metabolic activity (35).

We compared the mortality caused by wild-type isolates and the resulting individual *FKS2* mutant colonies in final-instar larvae of *G. mellonella* (Bichosa, Salceda de Caselas, Spain). Briefly, infecting inocula were prepared using a Neubauer chamber and checked in Sabouraud dextrose agar. Inocula ranging from 3×10^6 to 7×10^6 CFU per larva were accepted because the inoculum ranges did not affect the mortality of the larvae. Ten *G. mellonella* larvae per isolate were infected with 10 μl of a suspension of *C. glabrata*, and 2 control groups were established, one comprising 10 larvae inoculated with 10 μl of phosphate-buffered saline to monitor trauma and another comprising 10 noninjected larvae. Larvae were incubated at 37°C for up to 7 days after infection, and the number of dead larvae was recorded daily (50). Survival curves were obtained for wild-type isolates and the resulting individual echinocandin-resistant colonies using the Kaplan-Meier method (Graph Pad Prism 5.02 statistical software; GraphPad, La Jolla, CA, USA), and the differences were evaluated (log-rank test).

This study was approved by the Ethics Committee of Hospital Gregorio Marañón (CEIC-A1; study no. 208/16).

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6.1.4. Artículo 4: *MSH2* gene point mutations are not antifungal resistance markers in *Candida glabrata*.

En este estudio se evaluó la secuencia del gen *MSH2* en cepas de *C. glabrata* tanto sensibles como resistentes a las equinocandinas generadas *in vitro* e *in vivo*. Además, se determinó la relación entre las mutaciones en el gen *MSH2* y el genotipo, la adquisición de resistencia antifúngica, y el pronóstico del paciente.

La secuencia del gen *MSH2* se estudió en 124 aislados de *C. glabrata* que causaron episodios incidentes de candidemia (n=81), episodios secundarios de candidemia (n=9), endocarditis (n=2) y aislados resistentes a las candinas generados *in vitro* (n=32). El genotipado se realizó mediante microsatélites y "**m**ulti**l**ocus **s**equence **t**yping" (MLST). La sensibilidad antifúngica a las equinocandinas y al fluconazol se estudió mediante EUCAST.

Los aislados que causaron candidemia (n=90) fueron sensibles a las equinocandinas, cuatro de ellos fueron resistentes al fluconazol (CMI ≥64 mg/L), y un aislado de la válvula cardíaca fue resistente a micafungina y anidulafungina (CMI = 2 mg/L y 1 mg/L, respectivamente). Las mutaciones en el gen *MSH2* estuvieron presentes en el 44,4% de los aislados incidentes, siendo V239L la más frecuente, y no se correlacionaron con la resistencia antifúngica *in vitro* ni *in vivo*. Los microsatélites y el MLST revelaron 27 genotipos y 17 secuencias tipo, respectivamente, y los aislados resistentes a fluconazol no estuvieron genéticamente relacionados. La mayoría de cepas con genotipo idéntico mostraron las mismas mutaciones en el gen *MSH2*. No se encontraron diferencias clínicas entre los pacientes infectados por aislados con el gen *MSH2* de tipo salvaje y aislados con cualquier mutación puntual en este gen.

Se concluyó que la presencia de mutaciones en el gen *MSH2* en aislados de *C. glabrata* causantes de candidemia no se relacionó con la adquisición de resistencia antifúngica tanto *in vitro* como *in vivo*, con genotipos específicos, ni con los parámetros clínicos de los pacientes estudiados, incluido el uso de antifúngicos previo.

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MSH2 Gene Point Mutations Are Not Antifungal Resistance Markers in *Candida glabrata*

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ABSTRACT The high rates of antifungal resistance in *Candida glabrata* may be facilitated by the presence of alterations in the *MSH2* gene. We aimed to study the sequence of the *MSH2* gene in 124 invasive *C. glabrata* isolates causing incident episodes of candidemia ($n = 81$), subsequent candidemia episodes ($n = 9$), endocarditis ($n = 2$), and *in vitro*-generated echinocandin-resistant isolates ($n = 32$) and assessed its relationship with genotypes, acquisition of antifungal resistance *in vivo* and *in vitro*, and patient prognosis. The *MSH2* gene was sequenced, and isolates were genotyped using six microsatellite markers and multilocus sequence typing (MLST) based on six housekeeping genes. According to EUCAST, isolates causing candidemia ($n = 90$) were echinocandin susceptible, and four of them were fluconazole resistant (MIC ≥64 mg/liter). One isolate obtained from a heart valve was resistant to micafungin and anidulafungin (MICs, 2 mg/liter and 1 mg/liter, respectively). *MSH2* gene mutations were present in 44.4% of the incident isolates, the most common being V239L. The presence of *MSH2* mutations was not correlated with *in vitro* or *in vivo* antifungal resistance. Microsatellite and MLST revealed 27 genotypes and 17 sequence types, respectively. Fluconazole-resistant isolates were unrelated. Most *MSH2* mutations were found in cluster isolates; conversely, some mutations were found in more than one genotype. No clinical differences, including previous antifungal use, were found between patients infected by wild-type *MSH2* gene isolates and isolates with any point mutation. The presence of *MSH2* gene mutations in *C. glabrata* isolates causing candidemia is not correlated with specific genotypes, the promotion of antifungal resistance, or the clinical outcome.

KEYWORDS *Candida glabrata*, *MSH2* gene, antifungal resistance

Candida bloodstream infections have been increasing in recent years (1), posing a substantial economic burden for hospitals and high mortality among patients (2, 3). Since fluconazole resistance is becoming more common, echinocandins are currently preferred as first-line treatment (4). However, strains with resistance to echinocandins and/or to multiple antifungals have been sporadically reported (5).

The incidence of infections due to *Candida glabrata* has notoriously grown in Spain (1). In comparison to other *Candida* species, *C. glabrata* is characterized by the ability to develop fluconazole resistance and a certain degree of resistance to echinocandins in some hospitals (1, 6–9). In a previous work, we showed that *C. glabrata* can acquire *in vitro* resistance to echinocandins after exposure to low and/or increasing echinocandin concentrations (10–12). There is little information on why *C. glabrata* acquires resistance more rapidly to multiple antifungal classes than other *Candida* species. The potential for acquisition of resistance is unclear; however, organisms with a haploid

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genome, e.g., *C. glabrata*, might accumulate a number of mutations in key genes, such as *FKS*, during genome replication (13). DNA polymerase mismatches coupled with failures in DNA repair can lead to multiple antifungal resistance in *Candida* spp. (14, 15). An *MSH2* gene mismatch repair in *C. glabrata* and *Candida albicans* may lead to a hypermutable phenotype prone to developing antifungal resistance (14, 15). Furthermore, certain *C. glabrata* genotypes seem to harbor specific polymorphisms in the *MSH2* gene (16).

There are other aspects that require attention. First, only a small number of invasive isolates have been studied (16, 17). Second, studies have been reported without genotyping procedures (15, 17). Third, very few cases of *MSH2* gene sequences from antifungal-susceptible isolates that have shifted to resistant have been studied (15). Finally, the studies include scarce clinical data from infected patients. Here, we aim to study the sequence of the *MSH2* gene of a collection of *C. glabrata* isolates obtained in Spain causing fungemia and to assess the potential relationship with genotypes, possible acquisition of antifungal resistance *in vivo* and *in vitro*, and patient prognosis.

RESULTS

Antifungal susceptibility and *MSH2* gene sequencing. As per EUCAST breakpoints, the 90 blood culture isolates were echinocandin susceptible (geometric mean MICs of micafungin and anidulafungin, 0.015 and 0.023 mg/liter, respectively), and four of them (4.9%) were fluconazole resistant (patients 11, 20, 44, and 63; Fig. 1). Only one of the isolates, which was collected from the heart valve, was resistant to micafungin and anidulafungin (2 mg/liter and 1 mg/liter, respectively) and harbored an S663P mutation in hot spot 1 of the *FKS2* gene. The 21 *in vitro*-generated echinocandin-resistant colonies carried the following *FKS2* mutations: ΔF658, S663P, W715L, and E655A (12).

Thirty-six incident isolates from 81 patients with candidemia (44.4%) had one or more of the following *MSH2* gene point mutations: V239L, P208S, A942T, S653F, A313V, E456D, E459K, E7K, L588V, N890I, and/or L810H. Here, we report the S653F mutation for the first time. Mutation frequencies were 33.3% for V239L, 25.6% for P208S/N890I, 13.9% for V239L/A942T, 8.3% for E459K, and 2.7% for each of the following: S653F, A313V, E456D, E7K, L588V, N890I, and L810H. We observed a match between *MSH2* sequences of incident isolates and those causing second episodes. Two point mutations in the gene (P208S/N890I) were found in the four isolates from a patient who suffered two episodes of candidemia and endocarditis.

In our study, only two out of the four fluconazole-resistant isolates (from patients 11 and 20) harbored *MSH2* gene mutations (P208S/N890I and E456D, respectively) (Fig. 1). The sequence of the *in vitro*-generated echinocandin-resistant *MSH2* gene and the respective parent isolates were identical. Eight (38.1%) out of the 21 colonies that acquired *FKS2* mutations harbored *MSH2* gene mutations (P208S/N890I [$n = 4$], V239L [$n = 3$], and L810H [$n = 1$]). Nine (81.82%) out of 11 echinocandin-resistant colonies (i.e., those with an *FKS* mutation) had the same *MSH2* gene mutation (V239L) and were sourced from a single isolate, whereas the remaining two isolates were from different isolates and the *MSH2* wild type (Fig. 2).

Genotyping analysis and *MSH2* gene sequencing. Figure 1 shows the genotyping of the 81 incident isolates. Isolates responsible for second episodes, the case of endocarditis, and *in vitro*-generated isolates were isogenic to parent strains, regardless of the used method. Microsatellite markers revealed 27 genotypes (CG-1 to CG-27), 13 of which were clusters involving 82.7% of the isolates. Multilocus sequence typing (MLST) revealed 17 sequence types (STs), including six newly described ones (Table 1), and 11 STs being clusters that involved 92.6% of the isolates. The clusters with the highest number of isolates were microsatellites (CG-24 [30.86%], CG-6 [9.88%], and CG-23 [8.64%]) and MLST (ST3 [43.21%], ST34 [9.88%], ST10 [8.64%], and ST6 [7.41%]). In general, clusters detected by any method mostly matched, although there was a tendency for the microsatellites to split up ST clusters into clonally related genotypes

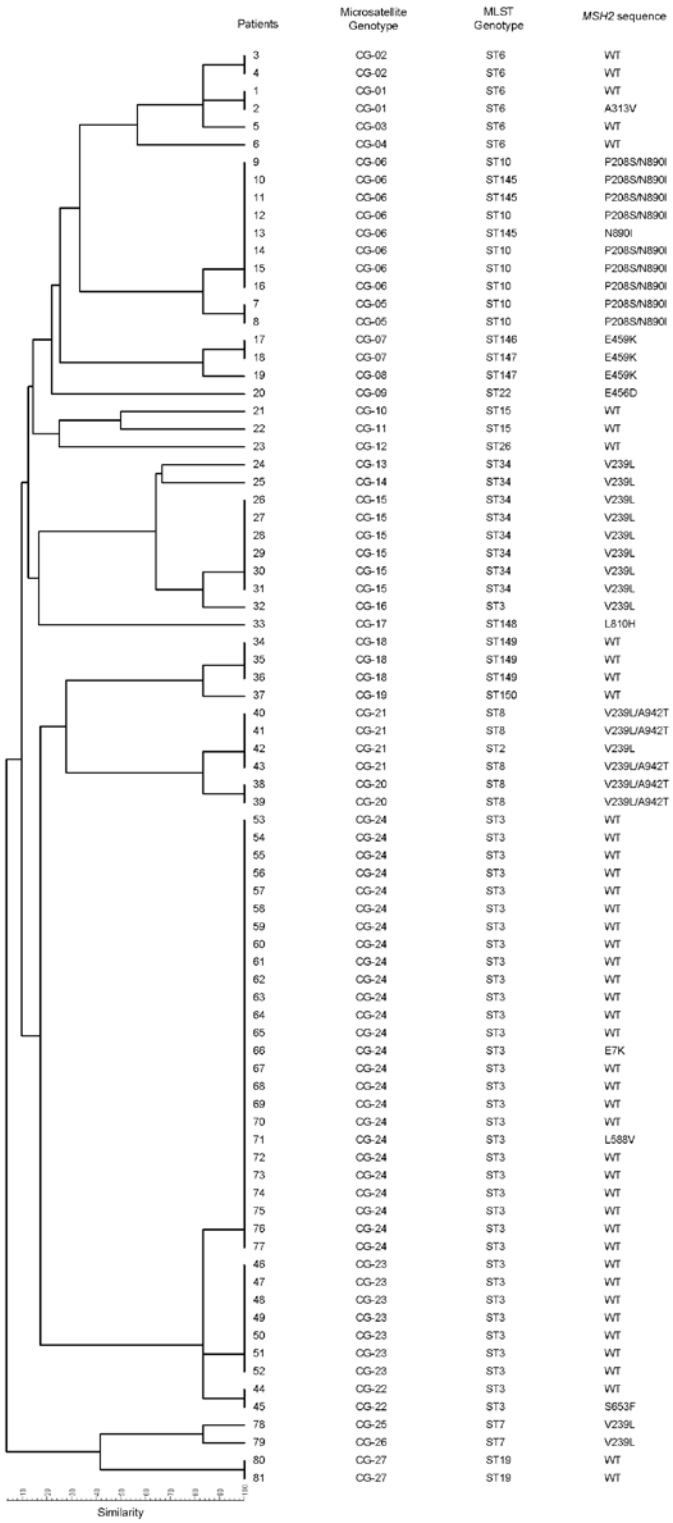

FIG 1 Genetic relationship with microsatellite markers between the incident 81 *Candida glabrata* isolates with the *MSH2* sequence and the sequence type (ST). WT, wild type.

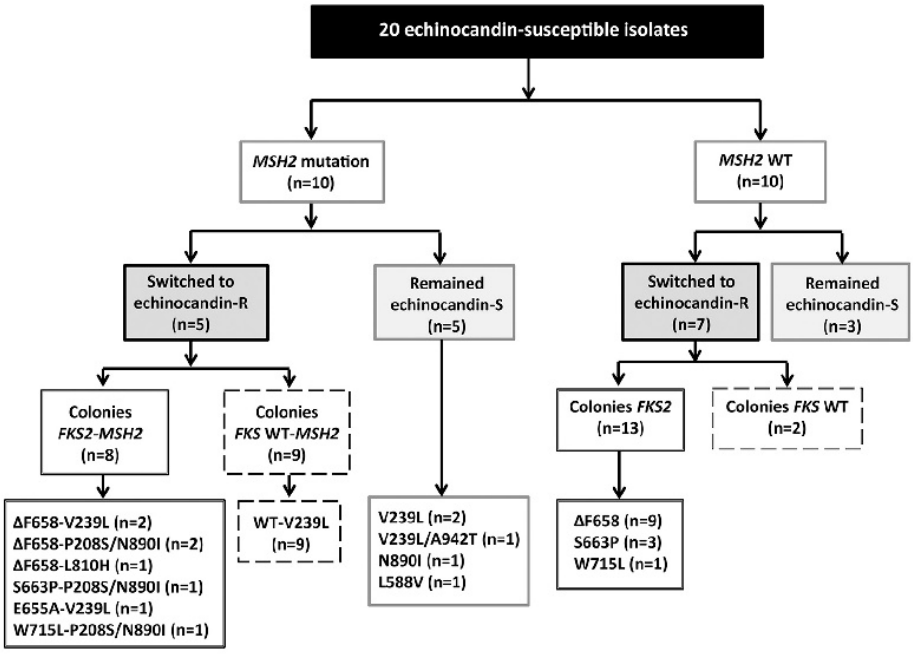

FIG 2 Description of the 20 echinocandin-susceptible isolates previously exposed *in vitro* to echinocandins and the generated colonies phenotypically resistant to echinocandins. R, resistant; S, susceptible; WT, wild type.

and, exceptionally, few microsatellite clusters were broken into clonally related sub-STs (Fig. 1). Fluconazole-resistant isolates were unrelated.

Most *MSH2* mutations were found in cluster isolates. In general, the bulk of isolates within each cluster showed the same *MSH2* polymorphism, although a few genotypes (CG-1, CG-22, and CG-24) involved wild-type and *MSH2* mutant isolates. Conversely, some mutations were found in more than one genotype (Fig. 1).

Association of *MSH2* mutations and genotypes with clinical characteristics of the patients. Table 2 summarizes the characteristics of the study patients. There were no differences in demographics, underlying conditions, risk factors for candidemia, or sources of candidemia between patients infected by wild-type *MSH2* isolates and those with any point mutation. Most patients infected with *MSH2* mutants were adults (94.4%), with a mean age of 71 years, and 63.9% were male. Gastrointestinal disease was the most frequent comorbidity in 50% of patients, and the most frequent department of admission was a medical ward (47.2%) (Table 2). Most cases had an intra-abdominal source (38.9%) or were central venous catheter-related infections (25%).

TABLE 1 Newly characterized STs and description of the alleles in the six housekeeping genes[a]

		Allele by gene					
Patient(s)	New ST	*FKS*	*LEU2*	*NMT1*	*TRP1*	*UGP1*	*URA3*
10, 11, 13	145	8	5	3	5	1	2
17	146	51	24	49	7	51	6
18,19	147	51	24	49	30	51	6
33	148	3	9	26	4	17	4
34, 35, 36	149	7	25	51	7	51	4
37	150	7	25	51	30	51	4

[a]ST 145 is a new combination of previously identified alleles. STs 146 to 150 are new combinations of previously identified alleles or the following new alleles (reported in this work for the first time).

TABLE 2 Demographics and clinical status of patients based on *MSH2* gene sequence[a]

Patient characteristic[b]	Total ($n = 81$)	Wild-type *MSH2* gene isolates ($n = 45$)	Isolates with *MSH2* gene mutations ($n = 36$)	*P* value
Demographics				
Age (median [IQR]) (yr)	71 (0–91)	72 (0–90)	71 (0–91)	0.556
Male	50 (61.7)	27 (60.0)	23 (63.9)	0.819
Neonate (≤28 days)	4 (4.9)	2 (4.4)	2 (5.6)	1
Department				
Medical	36 (44.4)	19 (42.2)	17 (47.2)	0.822
Surgical	21 (25.9)	12 (26.7)	9 (25.0)	1
Onco-hematology	3 (3.7)	1 (2.2)	2 (5.6)	0.582
ICU	21 (25.9)	13 (28.9)	8 (22.2)	0.612
Underlying disease				
Cardiovascular disease	32 (39.5)	18 (40.0)	14 (38.9)	1
Neurological disorder	15 (18.5)	10 (22.2)	5 (13.9)	0.398
Chronic renal failure	22 (27.2)	11 (24.4)	11 (30.6)	0.619
Diabetes mellitus	18 (22.2)	13 (28.9)	5 (13.9)	0.178
Pulmonary disease	19 (23.5)	9 (20.0)	10 (27.8)	0.440
Liver disease	13 (16.0)	8 (17.8)	5 (13.9)	0.765
Gastrointestinal disease	38 (46.9)	20 (44.4)	18 (50.0)	0.659
Solid-organ malignancy	28 (34.6)	14 (31.1)	14 (38.9)	0.490
Hematologic malignancy	2 (2.5)	1 (2.2)	1 (2.8)	0.694
Solid-organ transplant	1 (1.2)	0	1 (2.8)	0.444
HIV	5 (6.2)	2 (4.4)	3 (8.3)	0.651
Charlson index (median [IQR])	6 (0–12)	6 (0–10)	6 (0–12)	0.388
Risk factor				
Central venous catheter	64 (79.0)	35 (77.8)	29 (80.6)	0.791
Total parenteral nutrition	44 (54.3)	24 (53.3)	20 (55.6)	1
Chemotherapy	9 (11.1)	5 (11.1)	4 (11.1)	1
Neutropenia (<500 cells/mm^3)	2 (2.5)	1 (2.2)	1 (2.8)	1
Corticosteroids (prior mo)	15 (18.5)	8 (17.8)	7 (19.4)	1
Immunosuppressive therapy	4 (4.9)	3 (6.7)	1 (2.8)	0.625
Surgery	33 (40.7)	16 (35.6)	17 (47.2)	0.364
Abdominal surgery	26 (32.1)	13 (28.9)	13 (36.1)	0.633
Previous antimicrobial use	76 (93.8)	42 (93.3)	34 (94.4)	1
Antifungal use				
Previous AF used	17 (22.4)	9 (22.0)	8 (22.9)	1
Voriconazole	2 (2.5)	2 (4.4)	0	0.5
Fluconazole	15 (18.5)	7 (15.6)	8 (22.2)	0.567
Echinocandins	4 (4.9)	1 (2.2)	3 (8.3)	0.318
Amphotericin B	1 (1.2)	1 (2.2)	0	1
Duration of AF (days)	15 (3–63)	21 (9–44)	14.5 (3–63)	0.933
Time from previous AF to episode (days)	1 (−11–73)	0 (−6–73)	2.5 (−11–73)	0.755
Breakthrough fungemia	7 (8.6)	3 (6.7)	4 (11.1)	0.694
Fluconazole resistance	4 (4.9)	2 (4.4)	2 (5.6)	1
Clinical manifestation				
Septic shock	24 (29.6)	18 (40.0)	6 (16.7)	0.028
Pitt score (median [IQR])	1 (0–12)	2 (0–10)	0 (0–12)	0.093
Infection source				
Primary	14 (17.3)	9 (20.0)	5 (13.9)	0.562
Central venous catheter	27 (33.3)	18 (40.0)	9 (25.0)	0.235
Urinary tract	4 (4.9)	3 (6.7)	1 (2.8)	0.625
Abdomen	28 (34.6)	14 (31.1)	14 (38.9)	0.490
Other	3 (3.7)	0	3 (8.3)	0.084
Unknown	5 (6.2)	1 (2.2)	4 (11.1)	0.166
Therapeutic management				
No antifungals	14 (17.3)	9 (20.0)	5 (13.9)	0.562
Echinocandin as initial AF	29 (43.3)	15 (41.7)	14 (45.2)	0.809
Adequate targeted AF	62 (76.5)	34 (75.6)	28 (77.8)	1
Infection source control	56 (81.2)	31 (81.6)	25 (80.6)	1

(Continued on following page)

TABLE 2 (Continued)

Patient characteristic[b]	Total ($n = 81$)	Wild-type *MSH2* gene isolates ($n = 45$)	Isolates with *MSH2* gene mutations ($n = 36$)	*P* value
Outcomes				
Metastatic complications	13 (18.1)	6 (15.0)	7 (21.9)	0.543
ICU due to candidemia	10 (12.3)	6 (13.3)	4 (11.1)	1
7-day mortality	13 (16.0)	10 (22.2)	3 (8.3)	0.129
30-day mortality	24 (29.6)	18 (40.0)	6 (16.7)	0.028
Mortality >30 days	11 (13.6)	5 (11.1)	6 (16.7)	0.526
Overall mortality	35 (43.2)	23 (51.1)	12 (33.3)	0.121
Hospital length of stay (days)	26 (0–165)	25 (0–165)	26.5 (0–112)	0.711

[a]All data reported as number (%), unless otherwise specified.
[b]IQR, interquartile range; ICU, intensive care unit; HIV, human immunodeficiency virus; AF, antifungal.

Only one out of the four patients infected by fluconazole-resistant isolates was previously treated with fluconazole and caspofungin. The patient with endocarditis caused by the echinocandin-resistant isolate had previously received caspofungin, micafungin, and fluconazole. *In vitro* echinocandin-resistant-generated isolates came from 12 patients, of whom only four had received previous antifungal treatment. Eight patients with alterations in the *MSH2* gene isolates received systemic antifungals before the diagnosis, including fluconazole ($n = 8$) and echinocandins ($n = 3$). No statistical differences were determined in the median time of exposure to previous antifungals between patients infected by *MSH2* mutants and patients infected by wild-type isolates (14.5 and 21 days, respectively). Adequate targeted treatment and appropriate source control were equally ensured in both groups. Concerning clinical outcomes, there were no differences between groups with respect to metastatic complications, intensive care unit admission due to the severity of the candidemia, hospital length of stay, and early 7-day mortality. However, there was a higher proportion of patients with septic shock and 30-day mortality in those infected by wild-type *MSH2* gene isolates ($P = 0.028$) (Table 2). The same trend was observed when evaluating the mortality rates among patients with less severe clinical presentations (Pitt score, ≤ 1), although no statistical significance was found (data not shown). Furthermore, the ST3 genotype, which included mostly wild-type *MSH2* gene isolates (31/35), was detected more frequently in patients with septic shock (40% versus 21.7%; $P = 0.09$) and poor outcome (40% versus 21.7%; $P = 0.09$).

DISCUSSION

The analysis of a large number of *C. glabrata* isolates causing candidemia showed that the presence of *MSH2* gene mutations is not correlated with the previous use of antifungal agents, with specific genotypes (either defined using MLST or microsatellite markers), or with the promotion of antifungal resistance *in vitro* and in the patients.

Defects in DNA mismatch repair in multiple fungal species, including *C. glabrata*, have been reported to be associated with the acquisition of antifungal resistance (hypermutable phenotype) (14, 15, 18). A recent study identified a prevalent mutator *C. glabrata* phenotype caused by a mismatch repair defect in the *MSH2* gene (15). This phenotype may acquire resistance to multiple antifungals with the consequent added complication in patient care; the detection of *MSH2* point mutations was thus proposed as a marker of potential development of resistance. We did not find any correlation between the presence of *MSH2* gene mutations and the acquisition of antifungal resistance either *in vitro* or in clinical settings. This is in line with previous studies in which this lack of correlation was also highlighted (16, 17, 19).

A limitation of previous similar studies is the low number of resistant isolates. To solve this, in this study, we analyzed echinocandin-susceptible isolates that switched to a resistant phenotype after being exposed to candins *in vitro* ($n = 20$) (12) and naturally occurring in a patient with candidemia and endocarditis who had previously undergone treatment with echinocandins. We observed the following (Fig. 2): first, parent echinocandin-susceptible isolates and their respective resistant isolates had the same *MSH2* gene sequence. Second, echinocandin-susceptible isolates with either *MSH2*

gene mutations or a wild-type *MSH2* gene were able to switch to a resistant phenotype following drug exposure. Third, isolates with *MSH2* gene mutations were able to yield both echinocandin-resistant and echinocandin-susceptible isolates, as shown in the patient with endocarditis. Fourth, the *MSH2* gene sequence did not affect the acquired mechanism of resistance to echinocandins, as shown by the three isolates that were exposed to candins and yielded echinocandin-resistant isolates (*FKS2* mutants and wild-type *FKS*). Finally, mutations in the *MSH2* gene did not influence fluconazole resistance. The *MSH2* gene sequences of incident isolates and isolates causing second episodes were identical. Moreover, mutations were commonly found in incident susceptible isolates that did not turn resistant in further episodes. Here, the rate of isolates harboring mutations in the *MSH2* gene (44.4%) is similar to that reported in some other studies (15, 17) but lower than the 77.2% reported by Hou et al. (19). Certain mutations, such as V239L and P208S/N890I, have been associated with resistance (15, 17), whereas others that are less common, E7K, E456D, or E459K, have not (15–17).

The results of the two genotyping techniques are comparable, although a higher number of genotypes was obtained with microsatellite markers (19). Previous studies have tried to correlate specific genotypes with *MSH2* gene mutations (16, 19). Dellière and colleagues found a correlation between some microsatellite genotypes and *MSH2* gene mutations; however, the mutations were not genotype specific, as some of them were found in more than one genotype (16). Hou et al. reported a correlation between ST7 and ST10 and the presence of V239L and/or K583N and P208S/N890I mutations in the *MSH2* gene, respectively; however, the authors did not disclose if the same mutations were also found in other STs (19). Finally, the results described by Deshpande and Castanheira are in line with those of Hou et al., except that the V239L mutation was also found in ST2 and ST7, whereas ST3 isolates carried a wild-type *MSH2* gene (20). In considering the results of all these studies, including ours, there seems to be a relationship between STs and the *MSH2* gene sequence; e.g., ST7 and ST10 are found in different geographic places, including our isolates, and harbor specific mutations. However, there is no genotype specificity regarding the mutations; for example, we found that V239L was conspicuously present in ST2, ST3, ST7, ST8, and ST34 (Fig. 1). This suggests that a number of mutations may be potential *MSH2* gene constitutive polymorphisms rather than resistance-causing mutations. A recent study using whole-genome sequencing data suggests that these *MSH2* mutations are natural genetic variations rather than a mutator phenotype (21). Furthermore, *MSH2* gene sequences were mostly identical in the isolates belonging to a given ST or microsatellite genotype, with some exceptions (Fig. 1) (19, 22).

We report not only on the microbiological characterization of *C. glabrata* isolates but also the clinical description of the infected patients, which is a strength of our study. Patients infected by *C. glabrata* carrying *MSH2* gene mutations or with the wild-type *MSH2* gene presented similar demographic and clinical characteristics. Moreover, no differences in terms of previous antifungal use between groups were found. Our observations are in line with those from a previous study (16) but diverge with results from another work (15). Despite the comparable host characteristics and therapeutic management, a higher number of severe cases (higher median Pitt scores) was detected in the group of patients infected by strains with the wild-type *MSH2* gene, resulting in a higher incidence of septic shock and 30-day mortality. In fact, such differences could be related to the remarkable prevalence of ST3 genotype isolates among these patients. Other authors have reported that the most frequently found ST3 and ST7 may be correlated with a poor outcome (23), supporting our findings. In fact, our observations show that patients infected by ST3 show higher 30-day mortality than do patients infected by any of the other STs (40% versus 21.7%, respectively; $P = 0.09$). Further studies, including a higher number of episodes, should be carried out to evaluate the clinical impact of infections by ST3 isolates.

The low numbers of *FKS* mutant and fluconazole-resistant isolates found in the patients are in line with previous studies and the main limitation of our study (1, 24, 25).

However, our conclusions are supported by a number of *C. glabrata* resistant isolates *in vitro* generated from a set of incident isolates.

We conclude that the presence of *MSH2* gene mutations in *C. glabrata* isolates causing candidemia does not correlate with the previous use of antifungal agents by the patients, with specific genotypes, or with the promotion of antifungal resistance. Furthermore, not differences in clinical outcomes were observed.

MATERIALS AND METHODS

Yeast isolates, antifungal susceptibility, and patients. One hundred twenty-four *C. glabrata* isolates were studied. The distribution of isolates was as follows: isolates causing candidemia in 81 patients ($n = 81$ incident isolates and $n = 9$ second episodes; $n = 90$), isolates from the heart valve of one patient who developed endocarditis (patient no. 15, Fig. 1; $n = 2$), and a group of phenotypically echinocandin-resistant isolates that had been exposed *in vitro* (12) to echinocandins and were either *FKS2* mutants ($n = 21$) or wild-type *FKS* ($n = 11$) (Fig. 2). Second episode was defined as the isolation of *C. glabrata* from blood cultures taken ≥7 days after the diagnosis. Patients were admitted to Gregorio Marañón Hospital (Madrid, Spain) between January 2007 and December 2016, and isolates were molecularly identified and tested for susceptibility to micafungin, anidulafungin, and fluconazole following the EUCAST 7.3.1 microdilution procedure (26, 27). General characteristics (demographic, department of admission, underlying diseases, risk factors, and antifungal use) and clinical condition (clinical manifestation, source of infection, therapeutic management, and outcome) were collected, as well as the use of antifungals in the six months before the diagnosis.

***MSH2* sequencing and genotyping.** The *MSH2* gene of the 124 isolates was amplified and sequenced as described by Dellière et al. (16), with some modifications: 1.25 U of AmpliTaq gold (Applied Biosystems), 0.2 mM deoxynucleoside triphosphates, 2 mM $MgCl_2$, and 100 ng of the extracted DNA were used (12). *MSH2* sequences were compared with the reference wild-type *MSH2* gene sequence of *C. glabrata* (GenBank accession number XM_447585.1) (16).

The isolates were further genotyped by two previously described methods, a panel of six microsatellite markers (*MT1*, *ERG3*, *GLM4*, *CG10*, *CG7*, and *CG4*) (28–30) and MLST based on six housekeeping genes, including the loci *FKS*, *LEU2*, *NMT1*, *TRP1*, *UGP1*, and *URA3* (31). PCR products of the six housekeeping gene loci were sequenced in both directions. The combination of the alleles of the six loci defined an MLST genotype or sequence type (ST) according to the MLST database (http://pubmlst.org/cglabrata). Singleton genotypes were defined as those found only once, whereas a cluster was defined as the presence of ≥2 strains showing identical genotypes.

Data analysis. Normally distributed clinical variables are expressed as means ± standard deviation. Variables with nonnormal distribution are expressed as medians and interquartile ranges. Categorical variables are expressed as percentages and compared using Fisher's exact test or the χ^2 test, as appropriate. Continuous variables were compared with the Mann-Whitney U test. The comparisons were considered statistically significant with P values of <0.05 (IBM SPSS Statistics for Windows, version 21.0; Armonk, NY, USA).

Ethical considerations. This study was approved by the ethics committee of Hospital Gregorio Marañón (CEIC-A1; study no. 203/18).

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6.2. CAPÍTULO II: Determinación de parámetros cinéticos de crecimiento *in vitro* en aislados de *Candida* spp. causantes de candidemia.

6.2.1. Artículo 5: Growth kinetics in *Candida* spp.: Differences between species and potential impact on antifungal susceptibility testing as described by the EUCAST.

En este estudio se determinaron los parámetros cinéticos de crecimiento de aislados de *Candida* spp. causantes de candidemia, así como en cepas de *C. glabrata* tanto resistentes a las equinocandinas como al fluconazol, usando las condiciones de cultivo e incubación del propuestas en el método EUCAST EDef 7.3.1.

Se estudiaron 705 aislados clínicos de *Candida* spp. sensibles a los antifúngicos de pacientes con candidemia, 28 aislados de *C. glabrata* resistentes a las equinocandinas y 10 aislados de *C. glabrata* resistentes a fluconazol para calcular los parámetros cinéticos de crecimiento (la tasa de crecimiento promedio, el pico máximo, el tiempo hasta llegar a la tasa máxima de crecimiento y la fase de latencia). Se estudiaron las diferencias inter e intra especie, así como el porcentaje de aislados que alcanzaron una densidad óptica de 0,2 (mínimo requerido por EUCAST para poder obtener la CMI).

Se encontraron diferencias inter especies en la fase de crecimiento y en los parámetros cinéticos; *C. glabrata* fue la especie con un crecimiento más rápido y *C. parapsilosis* fue la especie con la fase de latencia más larga. Además, se encontraron variaciones dentro de la misma especie, determinándose una correlación positiva entre la tasa de crecimiento promedio y el pico máximo. Los aislados de *C. glabrata* resistentes a las equinocandinas tuvieron una tasa de crecimiento promedio significativamente más baja, en comparación con los aislados salvajes y resistentes al fluconazol. Periodos de incubación de 12 a 15 horas permitieron alcanzar el umbral de densidad óptica de 0,2 en el 100% de los aislados de *C. glabrata*, *C. tropicalis* y *C. krusei*.

Se concluyó que existían diferencias inter e intra especie en los parámetros cinéticos estudiados en los aislados clínicos de *Candida* spp. La resistencia a las equinocandinas en aislados de *C. glabrata* podría tener un impacto en la curva cinética de crecimiento. La susceptibilidad antifúngica mediante EUCAST podría anticiparse en ciertas especies, particularmente *C. glabrata*, *C. tropicalis* y *C. krusei*.

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Growth kinetics in *Candida* spp.: Differences between species and potential impact on antifungal susceptibility testing as described by the EUCAST

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Abstract

We studied the growth kinetic parameters of clinically relevant *Candida* species to verify the differences between species following the incubation and medium conditions recommended by the EUCAST. We analyzed 705 susceptible *Candida* spp. from patients with candidemia and *Candida glabrata* isolates resistant to echinocandins or fluconazole ($n = 38$) and calculated the average growth rate, maximum peak, time to maximum rate, and lag phase. We also examined inter- and intra-species differences, as well as the percentage of isolates reaching an optical density of 0.2 over time. Interspecies differences in growth phases and kinetic parameters were found. *C. glabrata* was the fastest growing species and the lag phase of *C. parapsilosis* was longer than that of the other species considered in this study. Strain-to-strain variations were found between species. A positive correlation between the average growth rate and maximum peak was determined. Echinocandin-resistant *C. glabrata* isolates had significantly lower average growth rate but higher time to maximum rate in comparison to wild-type *C. glabrata* isolates. Incubation periods of 12–15 hours allowed reaching the 0.2 optical density threshold in 100% of *C. glabrata, C. tropicalis*, and *C. krusei* isolates. We show differences in kinetic parameters between *Candida* spp. *C. glabrata* was the fastest growing species and *C. parapsilosis* showed the longest lag phase. Resistance to echinocandins may affect the growth kinetic curve. Speeding up antifungal susceptibility results could be possible for some isolates, particularly *C. glabrata*, *C. tropicalis*, and *C. krusei*.

Key words: *Candida* spp., growth curve, candidemia, kinetic parameters.

Introduction

The incidence of candidemia has shown an increasing trend in recent years[1] entailing significant economic cost for hospitals and high mortality for the patients.[1–4] The outcome of patients with candidemia is mainly influenced by the patient´s underlying and clinical conditions, as well as by how the infection is managed.[2]

However, intrinsic features of the strain causing the infection such as biofilm formation, antifungal resistance, or virulence may affect the prognosis of the patient.[5–9]

Another intrinsic characteristic of *Candida* isolates causing candidemia is their growth kinetic rate. The growth kinetic parameters are currently relatively easy to assess using automatic microtiter plate readers.[10] Furthermore, studying *Candida* growth kinetics can be useful for monitoring yeast optical density (OD) in the wells over time and potentially assess the MIC setting with shorter incubation periods (<24 hours) using the standard microdilution methodology developed by the EUCAST.[11]

Studies on bacterial growth rate of strains causing infections have been carried out with specific populations (e.g., patients with cystic fibrosis)[12,13] for monitoring antibacterial activity[14] or the inhibition of biofilm formation,[15] or to study bacterial fitness.[16–18] However, little attention has been given to the study of these parameters in *Candida*.

In this study, we aim to assess the growth kinetic parameters among clinically relevant *Candida* species isolates causing candidemia to determine potential differences between species. We will also mimic the incubation and medium conditions described in EUCAST EDef 7.3 to analyze fungal growth over time of incubation.

Methods

Isolates and antifungal susceptibility

We studied 705 fluconazole- and echinocandin-susceptible yeast strains (*C. albicans* sensu stricto [$n = 351$], *C. parapsilosis* sensu stricto [$n = 200$], *C. glabrata* sensu stricto [$n = 83$], *C. tropicalis* [$n = 54$], *C. krusei* [$n = 17$]) from patients with candidemia (one isolate per patient) admitted to Gregorio Marañón Hospital (Madrid, Spain) between January 2007 and December 2017. Furthermore, *C. glabrata* sensu stricto isolates resistant to echinocandins ($n = 28$) or fluconazole ($n = 10$) obtained in previous studies were also tested.[19–22] The isolates were identified by molecular analysis and tested for susceptibility to micafungin, anidulafungin, and fluconazole following the microdilution procedure developed by EUCAST.[11,23]

In vitro growth rate determination

The *in vitro* growth kinetics of all isolates were analyzed as described elsewhere.[20,24] Inoculum conditions and incubation media were similar to those recommended by EUCAST E.Def 7.3.1. Briefly, 100 μl of the adjusted inoculum (1×10^5 to 5×10^5 cells/ml) of each isolate was added to 100 μl of double-concentrated Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 2% glucose (Merck KGaA, Darmstadt, Germany) and MOPS (Sigma-Aldrich, Co., St. Louis, MO, USA) in flat-bottom 96-well microdilution trays (Grenier® Ref. 655161, Greiner bio-one, Frickenhausen, Germany). All isolates were tested in triplicate. Yeast growth curves were obtained by incubating the trays at 35°C with moderate shaking in a spectrophotometer (Thermo Scientific, Madrid, Spain); the OD in each well was measured every 15 minutes (up to a maximum of 36 hours) at 490 nm. The following kinetic parameters were calculated: average growth rate (average increase in sample absorbance from time 0 to 36 hours, s^{-1}), maximum peak (maximum absorbance measurement in the curve, OD, arbitrary units), time to maximum rate (time at which maximum rate [maximum increment between two consecutive OD readings] occurs), and lag phase (time required to start the exponential phase of growth, no increase in OD, hours).[10,25]

Data analysis

Average growth rate, maximum peak, and time to maximum rate (SkanIt Software for Multiskan FC, Thermo Scientific, Madrid, Spain), and lag phase (Graph Pad Prism 5.02 statistical software; GraphPad, La Jolla, CA, USA) for individual isolates were retrieved and the median of three separate experiments per isolate was calculated. For each curve, an R^2 for the adjustment of the curve was obtained. Differences in kinetic parameters among species and between susceptible and resistant *C. glabrata* isolates were studied by using non-parametric tests, Kruskal-Wallis and Mann–Whitney; the comparisons were considered statistically significant with P values $< .05$ (IBM SPSS Statistics for Windows, version 21.0; Armonk, NY, USA).

Kinetic parameters of the isolates, expressed as median and interquartile range, were compared by using the Spearman correlation coefficient.

Representation of the median OD and interquartile range for the various *Candida* species per hour of incubation (up to 36 hours) was used to generate fungal growth figures. The percentage of *Candida* spp. isolates reaching an OD of 0.2 every hour of incubation is presented.

Ethical considerations

This study was approved by the Ethics Committee of Hospital Gregorio Marañón (CEIC-A1; study no. 192/18).

Results

Analysis of susceptible isolates

Growth curves of the five *Candida* spp. allowed identifying interspecies differences of the lag, exponential, and stationary phases between the various study species (Fig. 1).

Significant differences were seen in the kinetic parameters (Table and Fig. 2). The average growth rate and maximum peak of *C. albicans* and *C. parapsilosis* was significantly lower than that of the remaining species ($P < 0.01$). Significant differences in time to maximum rate were found between *C. parapsilosis* and *C. glabrata* ($P < .001$), between *C. parapsilosis* and *C.*

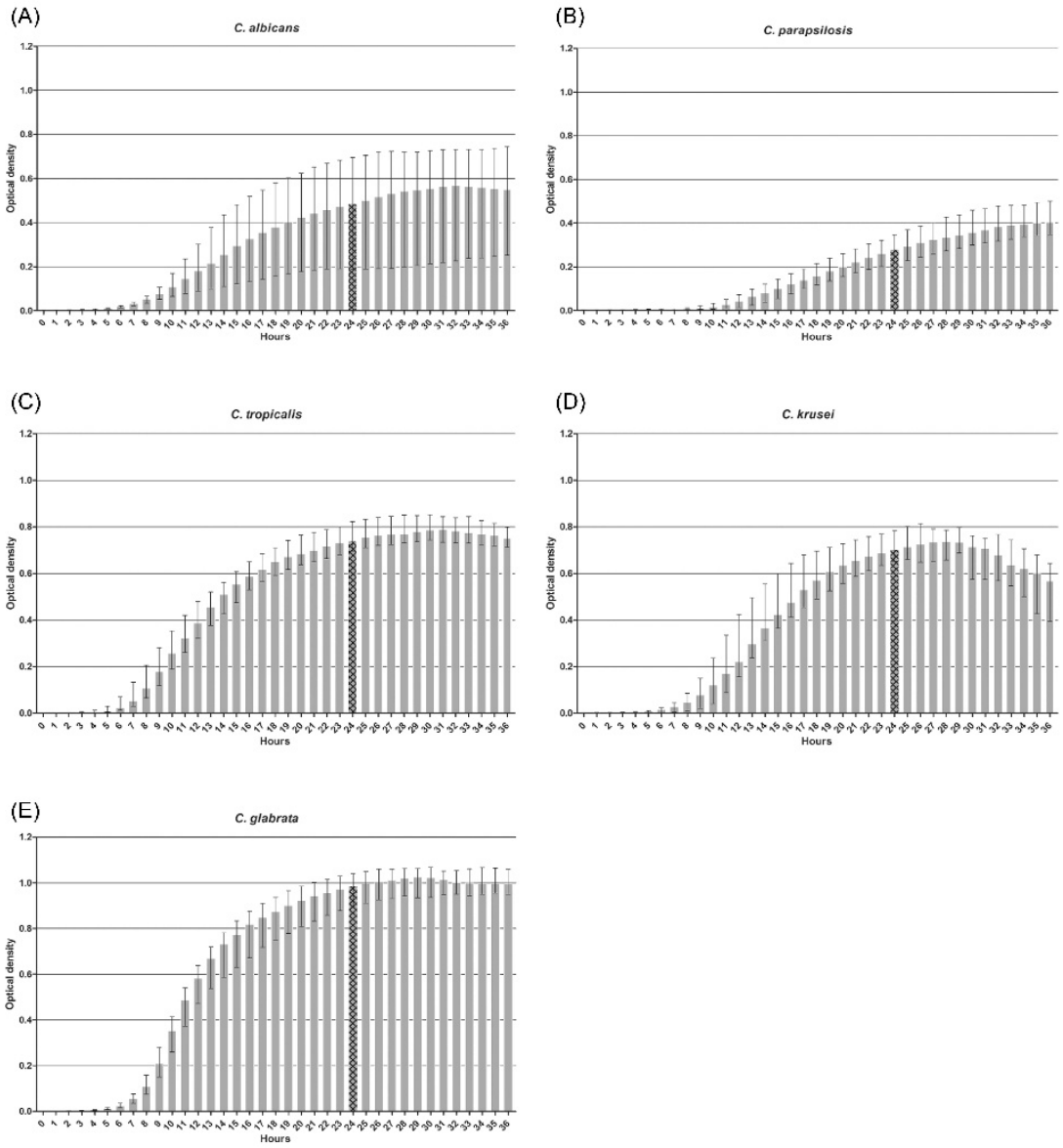

Figure 1. Kinetic growth curves of different antifungal-susceptible *Candida* spp. A, *C. albicans*; B, *C. parapsilosis*; C, *C. tropicalis*; D, *C. krusei*; E, *C. glabrata*. The median of the optical density and interquartile range over time of incubation.

tropicalis ($P < .001$), and between *C. glabrata* and *C. albicans* ($P < .001$). Finally, the lag phases were significantly different between the various species (*C. parapsilosis* vs. *C. glabrata*; *C. parapsilosis* vs. *C. albicans*; *C. parapsilosis* vs. *C. tropicalis*; *C. tropicalis* vs. *C. krusei*) ($P < .05$). Based on these results, *C. glabrata* was the fastest growing species, whereas *C. albicans* and *C. parapsilosis* were the slowest (Table and Fig. 2). The lag phase of *C. parapsilosis* was notably longer compared to that of the other study species, indicating that *C. parapsilosis* initiates the exponential growth phase later (Table and Fig. 2). However, many strain-to-strain variations in the kinetic parameters were observed within a single species (Fig. 1 and 2). According to Spearman correlation coefficient, a moderate positive correlation ($P < 0.05$) between the average growth rate and the maximum peak in all species was found (*C. albicans*, 0.43; *C. parapsilosis*, 0.38; *C. tropicalis*, 0.52; *C. krusei*, 0.50; and *C. glabrata*, 0.55).

Table. Median and interquartile range of kinetic parameters for susceptible and resistant species.[a]

	Species	No. of isolates	Average growth rate (seconds^{-1})	Maximum peak (optical density)	Time to maximum rate (seconds)	Lag phase (hours)
Susceptible	*Candida albicans*	351	3.87×10^{-6} (2.9–4.5 $\times 10^{-6}$)	0.55 (0.45–0.65)	9.74×10^{4} (5.1–13.7 $\times 10^{4}$)	7.60 (6.7–8.8)
	Candida parapsilosis	200	4.15×10^{-6} (3.3–5.1 $\times 10^{-6}$)	0.40 (0.28–0.57)	9.19×10^{4} (7.9–11.6 $\times 10^{4}$)	10.57 (9.09–12.55)
	Candida tropicalis	54	6.84×10^{-6} (6.3–7.6 $\times 10^{-6}$)	0.78 (0.72–0.95)	8.03×10^{4} (5.5–9.7 $\times 10^{4}$)	7.39 (6.7–8.1)
	Candida krusei	17	6.33×10^{-6} (5.2–6.9 $\times 10^{-6}$)	0.73 (0.67–0.82)	9.05×10^{4} (6.5–9.9 $\times 10^{4}$)	8.89 (8.28–10.56)
	Candida glabrata	83	7.80×10^{-6} (6.0–8.8 $\times 10^{-6}$)	1.01 (0.96–1.19)	6.25×10^{4} (3.7–7.1 $\times 10^{4}$)	7.42 (7.14–8.44)
Fluconazole-resistant	*Candida glabrata*	10	7.95×10^{-6} (6.1–9.3 $\times 10^{-6}$)	1.01 (0.92–1.15)	6.43×10^{4} (5.3–7.3 $\times 10^{4}$)	8.57 (7.83–9.16)
Echinocandin-resistant	*Candida glabrata*	28	4.70×10^{-6} (3.6–5.7 $\times 10^{-6}$)	1.13 (1.06–1.22)	1.04×10^{5} (7.2–13.3 $\times 10^{4}$)	8.73 (7.62–8.86)

[a]Significant differences by Kruskal-Wallis test were found in all the kinetic parameters ($P < 0.001$).

The species with the best correlation coefficient was *C. glabrata*, followed by *C. tropicalis* and *C. krusei*. This positive correlation was also observed within isolates in each specie and isolates with a higher average growth rate had a higher maximum peak. No associations among the other kinetic parameters were detected.

Analysis of resistant isolates

We studied the kinetic parameters of fluconazole- and echinocandin-resistant *C. glabrata* isolates and compared them against wild-type *C. glabrata* isolates (Table). No significant differences were found between fluconazole-resistant and wild-type isolates. However, echinocandin-resistant *C. glabrata* isolates had significantly lower average growth rate but higher time to maximum rate in comparison to wild-type *C. glabrata* isolates ($P < 0.001$) (Table).

Optical density over time

Median OD and interquartile range for different *Candida* spp. growth curves over time are shown in Figure 1. Depending on the species, the required minimum OD value (0.2) was achieved at different time points (Fig. 1), as follows: *C. tropicalis* (10 hours), *C. glabrata* (9 hours), *C. krusei* (12 hours), *C. albicans* (13 hours), and *C. parapsilosis* (20 hours). However, there were considerable variations due to interquartile range. For that reason, we calculated the percentage of isolates within a species that reached the 0.2 OD over time (Fig. 3).

The OD = 0.2 cut-off was not reached in 100% of *C. albicans* and *C. parapsilosis* isolates at the incubation time point recommended by EUCAST (24 hours); 100% of *C. parapsilosis* isolates reached the OD cut-off after 35 hours of incubation whereas *C. albicans* isolates did not even at that time point reading (Fig. 3). The OD readings were above 0.2 in 100% of *C. glabrata*, *C. tropicalis*, and *C. krusei* isolates after 12–15 hours of incubation (Fig. 3).

Discussion

To our knowledge, this is the first study showing differences in fungal growth kinetic patterns in a large collection of *Candida* spp. isolates causing candidemia and considerable strain-to-strain variations within species.

C. glabrata grew vigorously, showed the steepest kinetic curve, and was the fastest species in terms of average growth rate and maximum OD achieved in the medium and incubation conditions used in this study (Fig. 1). Since similar inoculum for all isolates were spiked on the microtiter plate, the differences in the kinetics among species may be explained by the high number of *C. glabrata* cells per unit of volume in the wells compared to other species. This would result from either a smaller cell size of *C. glabrata* and/or short germination time (reduced time needed to duplicate its haploid genome). The remaining species showed slower average growth rate with *C. tropicalis* and *C. krusei*

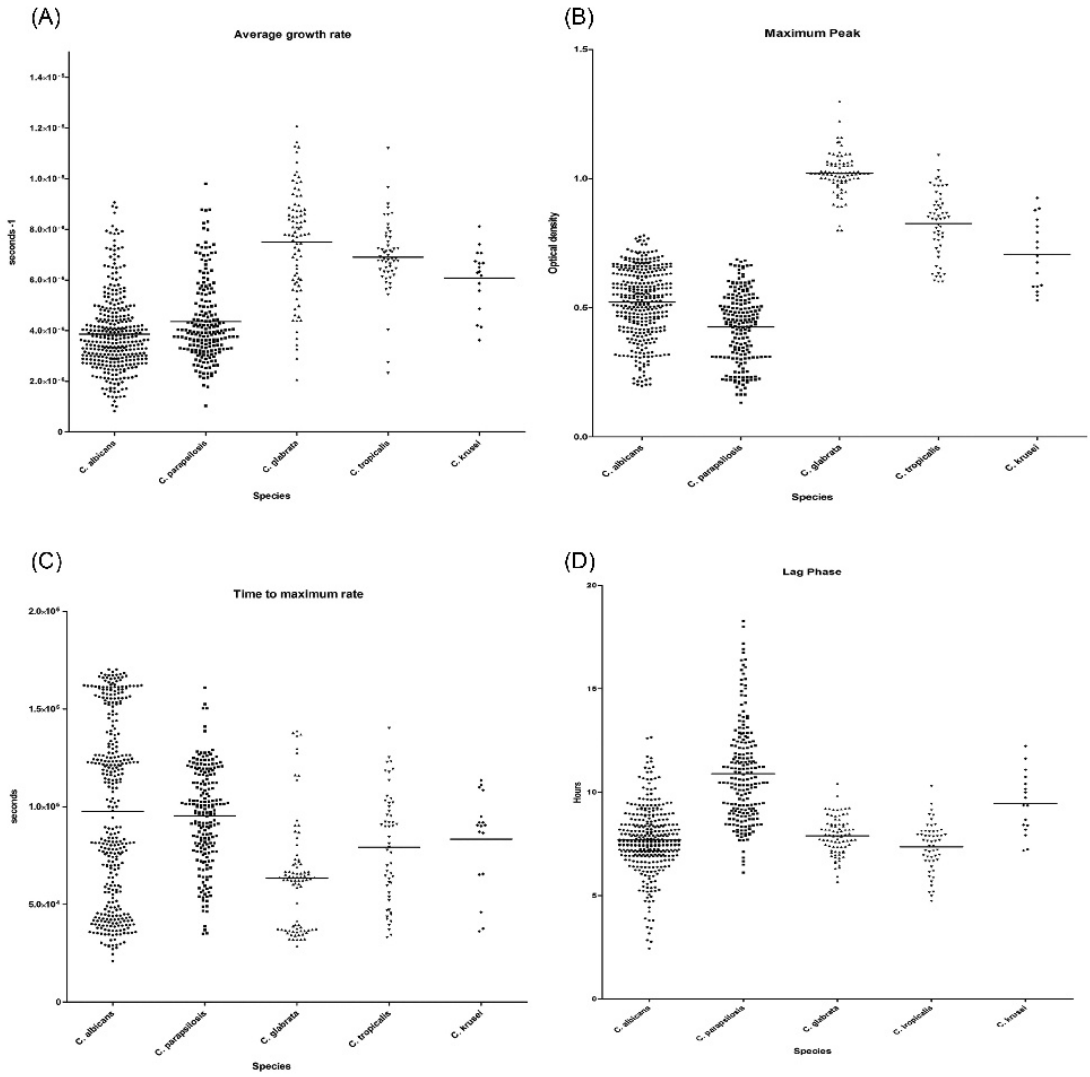

Figure 2. Kinetic parameters of antifungal-susceptible isolates of each of the five studied *Candida* species (A, average growth rate; B, maximum peak; C, time to maximum rate; D, lag phase). Median of each parameter per species is shown.

being the second and the third fastest species, respectively, and *C. parapsilosis* and *C. albicans* being the slowest species. Resistance to fluconazole in *C. glabrata* did not affect fungal growth kinetics. However, echinocandin-resistant *C. glabrata* isolates showed slower average growth rate and longer time to maximum rate than echinocandin-susceptible *C. glabrata* isolates. However, this issue is controversial since some authors have reported that *FKS* mutations in both *C. albicans* and *C. glabrata* does not have a clear impact on the growth kinetics.[24,26] These observations suggest potential strain-to-strain variations in the fitness of *FKS* mutant isolates.

The opposite of the above described is observed when blood cultures of patients with candidemia are studied since time to positivity of blood cultures is significantly longer for *C. glabrata* in comparison with other species.[27–30] In contrast, putting the remaining four species in a trot, the order mirrored the one found in the blood cultures in automatic systems. *C. tropicalis* and *C. krusei* were usually the species with the shortest time to positivity, followed by *C. parapsilosis* and *C. albicans* (with similar times).[27,28] Previous studies have reported that the time to positivity of blood culture bottles in automated systems is affected by the fungal inoculum, bottle type (aerobic vs. anaerobic

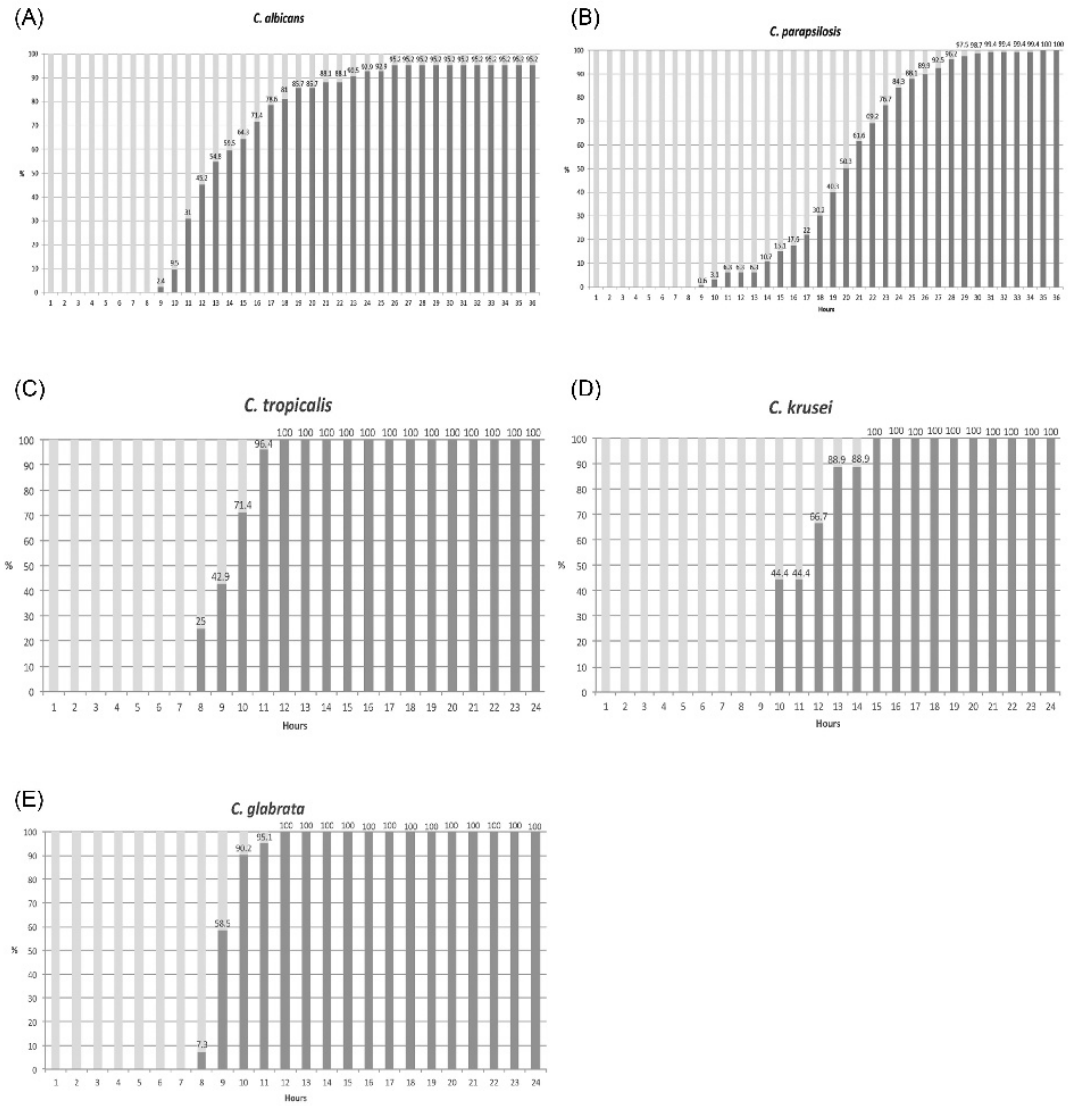

Figure 3. Percentage of antifungal-susceptible *Candida* spp. isolates that reach an optical density ≥ 0.2 over time of incubation. A, *C. albicans*; B, *C. parapsilosis*; C, *C. tropicalis*; D, *C. krusei*; E, *C. glabrata*.

conditions), and/or the medium composition.[29–33] The higher the fungal load, the shorter the time to positivity for all species regardless the type of bottle, with the exception of *C. glabrata* that seems to grow better in Mycosis IC/F bottles even with low inoculum[30]. Furthermore, *C. glabrata* grows preferentially in anaerobic vials; in fact, early growth in anaerobic vials is useful for predicting the presence of *C. glabrata* in the bottle as previously reported.[29,31,32]

The experimental conditions in our study cannot be compared to the environment where the yeast in the blood cultures are in terms not only of inoculum but also of medium composition and nutrient availability. Unfortunately, the composition of the BACTEC medium is mostly unknown. Furthermore, *Candida* cfu/ml extracted from blood (venipuncture) and then inoculated in blood culture bottles cannot be assessed, but it is much lower than the inoculum used in this study. However, it is well known that the combination medium composition-inoculum affects the kinetic curves.[25,34,35] In order to study if the inoculum may lead to differences in kinetics, 10 randomly selected isolates per species, including the echinocandin- and fluconazole-resistant

C. glabrata isolates, were analyzed using different adjusted inocula ($1–5 \times 10^4$, $1–5 \times 1\ 0^3$, $1–5 \times 10^2$, $1–5 \times 10^1$ cells/ml). Kinetic parameters decreased and the lag phase was delayed with lower inoculum, whereas differences across species remained unchanged (data not shown).

The experimental conditions used in this study—culture medium, inoculum, and temperature of incubation—were exactly the same as in the EUCAST EDef 7.3.1 protocol, mimicking the growth control drug-free well over time. Relevant information for MIC determination can be extracted after 24 ± 2 hours of incubation. First, the morphology of the kinetic curves at 24 hours allows detecting when *C. parapsilosis* is in the exponential phase, whereas the remaining species are in a deceleration (prestationary) phase where cell replication is probably decreasing as a consequence of competition, restrictions in nutrient availability and space.[36] Second, the growth kinetic curve between 22 hours and 26 hours shows similar results except for *C. krusei*. In the latter case, the curve evolves from the prestationary phase (22 hours) to a post-stationary phase (26 hours) where cannibalism can be presumed. Since the MIC is determined after the end of the exponential phase, no big differences in OD were found between 22 hours and 26 hours of incubation. However, the considerable strain-to-strain variations found within a species could imply that isolates are performed. The MIC of azoles and echinocandins against *Candida* is defined as the minimum concentration of drug leading to ≥50% decrease of fungal growth respect to the drug-free growth control; it means that different amounts of cells in the control well may lead to different MIC determinations. However, we do not know to what extent the growth curve over time is affected by the presence of antifungals. Further studies should be carried out to assess the impact kinetic curve phases have on MIC determination.

Since antifungal resistance detection may be useful to correct and optimize antifungal treatment, speeding up antifungal susceptibility reports would be welcome. OD of the drug-free well needs to reach a minimum of 0.2 value after 24 ± 2 hours of incubation.[11] However, this threshold can be reached earlier or additional incubation may be required. The earlier the threshold is reached, the shorter the incubation time will be. Very few isolates reached this threshold after 8–10 hours of incubation. However, the incubation times required by most isolates (80%) ranged between 10 hours (*C. glabrata*) to 24 hours (*C. parapsilosis*). Thus, speeding up antifungal susceptibility reports may be feasible only in some isolates, particularly those of *C. glabrata*, *C. tropicalis*, and *C. krusei*. There are certain considerations to be taken into account. First, speeding up results only a few hours compared to the regular reading at 24 hours may not have a clinical impact. Second, anticipating readings would invariably lead to set the MIC in the exponential phase of the fungal growth and the impact of this phenomenon is unknown. Finally, future studies should prove that yeast growth in presence of antifungals allows result interpretation with shorter incubation times.

In conclusion, we show differences in the kinetics parameters among *Candida* spp. *C. glabrata* was the fastest growing species and *C. parapsilosis* showed the longest lag phase. Resistance to echinocandins could have an impact on the growth kinetic curve. Speeding up antifungal susceptibility can be possible for certain isolates, particularly *C. glabrata*, *C. tropicalis*, and *C. krusei*.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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6.3. CAPÍTULO III: Detección rápida de resistencia a equinocandinas en *Candida* spp.

6.3.1. Artículo 6: Resistance to echinocandins in *Candida* can be detected by performing the Etest directly on blood culture samples.

En este estudio se evaluó el papel del Etest® y de las placas de agar con anidulafungina realizados directamente sobre hemocultivos positivos como técnicas rápidas de detección de resistencia a las equinocandinas en *Candida* spp.

Se estudiaron 80 hemocultivos positivos de pacientes con candidemia: 60 aislados de *Candida* spp. sensibles a las equinocandinas (*C. albicans* [n = 20], *C. tropicalis* [n = 20], *C. glabrata* [n = 20]) y 20 aislados de *C. parapsilosis* con sensibilidad intermedia a las equinocandinas. Adicionalmente, se inocularon botellas de hemocultivos con 35 aislados de *Candida* spp. resistentes a la equinocandinas. Un total de 3-6 gotas del medio de cultivo de cada botella positiva se aplicó directamente sobre placas de agar RPMI 1640 con tiras de Etest® de micafungina y anidulafungina (ET_{DIR}) y en placas de agar Sabouraud con 2 mg/L de anidulafungina. Todas las placas se incubaron a 35ºC durante 24 horas. Además, los aislados fueron testados mediante EUCAST y Etest® estándar (ET_{SD}) para calcular el acuerdo esencial y categórico entre los métodos.

El acuerdo esencial y el categórico entre el método EUCAST, ET_{DIR} y ET_{SD} fue >97%. El acuerdo esencial entre EUCAST y ET_{DIR} para ambas candinas fue >97%. El acuerdo categórico entre la secuencia de los genes *FKS* y ET_{DIR} fue del 97%. Las CMIs de anidulafungina y de micafungina obtenidas por ET_{DIR} (≥0,19 mg/L y ≥0,064 mg/L, respectivamente) separaron los aislados sensibles/salvajes de los resistentes/mutantes. El acuerdo categórico entre el método de EUCAST y el crecimiento en las placas con anidulafungina fue bajo (63%) con la excepción de *C. glabrata* (94%).

Se concluyó que el Etest® de micafungina y anidulafungina realizado directamente sobre hemocultivos positivos de pacientes con candidemia era un procedimiento fiable y rápido para detectar resistencia a las equinocandinas en *Candida* spp., siendo un método fácilmente asumible en la rutina del laboratorio de microbiología.

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Resistance to Echinocandins in *Candida* Can Be Detected by Performing the Etest Directly on Blood Culture Samples

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ABSTRACT We examined the rapid evaluation of susceptibility to echinocandins in *Candida* spp. using the Etest performed directly on positive blood cultures and anidulafungin-containing agar plates. We prospectively collected 80 positive blood cultures (Bactec-FX system, Becton-Dickinson, Cockeysville, MD, USA) with echinocandin-susceptible *Candida* spp. ($n = 60$) and echinocandin-intermediate *Candida parapsilosis* ($n = 20$) from patients with candidemia. Additionally, blood culture bottles of nonfungemic/bacteremic patients were spiked with 35 echinocandin-resistant *Candida* species isolates. A total of 2 to 4 drops of medium from each bottle were stroked directly onto both RPMI 1640 agar plates with micafungin and anidulafungin Etest strips (ET_{DIR}) and Sabouraud agar plates containing 2 mg/liter of anidulafungin. The isolates were tested according to the EUCAST method and Etest standard (ET_{SD}). Essential and categorical agreement between the methods was calculated. The essential agreement and categorical agreement between the EUCAST method and ET_{DIR} and ET_{SD} were both >97.4%. The essential agreement between ET_{DIR} and the EUCAST method for both echinocandins was >97%. The categorical agreement between the *FKS* sequence and ET_{DIR} was 97.4%. The ET_{DIR} MICs of anidulafungin and micafungin (≥0.19 mg/liter and ≥0.064 mg/liter, respectively) effectively separated all susceptible *FKS* wild-type isolates from the resistant *FKS* mutant isolates. The categorical agreement (62.6%) between the EUCAST method and growth on anidulafungin-containing plates was poor, with the best agreement observed for *Candida glabrata* (94.2%). When performed directly on positive blood cultures from patients with candidemia, the Etest with micafungin and anidulafungin is a reliable procedure for the rapid testing of susceptibility to echinocandins in *Candida* species isolates.

KEYWORDS *Candida* spp., EUCAST procedure, Etest, echinocandins

The incidence of invasive fungal infections has increased in many institutions, and mortality rates soar when an appropriate antifungal treatment is delayed (1–4). Echinocandins are recommended as the first-line treatment for invasive candidiasis (5, 6). Although the rates of resistance to echinocandins remain low (1, 7–10), recent publications are alerting physicians to an increased rate of resistance in some geographic areas (11, 12). Resistance is associated with a poor prognosis in patients with candidemia treated with echinocandins; consequently, detection may help to optimize antifungal treatment (11, 13). Echinocandin resistance can be detected in the clinical microbiology laboratory using broth microdilution methods, such as EUCAST and CLSI reference methods, or commercial methods, such as with Sensititre YeastOne, Vitek,

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TABLE 1 Distribution of MICs and geometric mean MICs of micafungin and anidulafungin obtained using EUCAST, ET_{SD}, and ET_{DIR} methods

Drug	Species (no. of isolates)	Method	MIC (mg/liter)[a]										
			GM[b]	0.015	0.032	0.064	0.125	0.25	0.5	1	2	4	≥8
Micafungin	*C. albicans* (21)	EUCAST	0.018	20						**1**			
		ET_{DIR}	0.021	20									**1**
		ET_{SD}	0.021	20									**1**
	C. parapsilosis (20)	EUCAST	0.841					1	7	8	4		
		ET_{DIR}	1.149						2	12	6		
		ET_{SD}	1						4	12	4		
	C. tropicalis (23)	EUCAST	0.033	9	11			**2**			**1**		
		ET_{DIR}	0.039	6	14			**1**	**1**			**1**	
		ET_{SD}	0.042	3	17			**1**	**1**		**1**		
	C. glabrata (51)	EUCAST	0.192	20		**6**		**1**	**1**	**5**	**8**	**10**	
		ET_{DIR}	0.224	19	1	**1**	**4**	**2**	**1**	**6**	**8**	**5**	**4**
		ET_{SD}	0.178	20		**1**	**5**	**1**	**5**	**5**	**10**	**3**	**1**
Anidulafungin	*C. albicans* (21)	EUCAST	0.016	18	2			**1**					
		ET_{DIR}	0.021	20									**1**
		ET_{SD}	0.021	20									**1**
	C. parapsilosis (20)	EUCAST	1.866						1	4	11	4	
		ET_{DIR}	2.928							1	7	12	
		ET_{SD}	3.031							1	6	13	
	C. tropicalis (23)	EUCAST	0.023	18		4				**1**			
		ET_{DIR}	0.038	7	12	1		**1**	**1**		**1**		
		ET_{SD}	0.029	15	5			**1**	**1**		**1**		
	C. glabrata (51)	EUCAST	0.258	8	12			**4**	**4**	**6**	**13**	**4**	
		ET_{DIR}	0.311	16	4			**1**	**5**	**2**	**10**	**12**	**1**
		ET_{SD}	0.239	20				**3**	**5**	**5**	**12**	**4**	**2**

[a]The MICs obtained by the ET_{SD} and ET_{DIR} were increased to the concentration of the next 2-fold dilution matching the micafungin dilution scale used for the EUCAST procedure. Numbers in bold indicate resistant isolates.
[b]GM, geometric mean.

disk diffusion, or plastic gradient strips. Alternatively, mutations in *FKS1* and *FKS2* (*Candida glabrata* only) can be detected using molecular techniques (12, 14, 15).

Phenotypic procedures for *Candida* antifungal susceptibility testing require pure-cultured isolates and are hindered by a slow turnaround time (48 to 72 h from the diagnosis of candidemia). We previously reported that the Etest performed directly on positive blood samples for yeasts was comparable to the standard CLSI and EUCAST approaches for the detection of both wild-type and azole-resistant *Candida* species isolates within 24 h of the diagnosis (2, 16). Unfortunately, given the lack of resistant isolates, we were unable to study the role of the procedure in the detection of the resistance of *Candida* to echinocandins.

On the basis of a set of echinocandin-resistant *Candida* isolates, we assessed the role of the Etest performed directly on artificially spiked blood cultures for the detection of resistance to echinocandins. Anidulafungin-containing agar plates were also tested for the screening of resistant isolates.

(This study was partially presented at the 28th European Congress of Clinical Microbiology and Infectious Diseases in Madrid, Spain, 2018 [17].)

RESULTS

The antifungal activities of micafungin and anidulafungin determined by EUCAST, Etest strips (ET_{DIR}), and Etest standard (ET_{SD}) procedures against the 115 isolates are shown in Table 1. The geometric mean (GM) MICs obtained by the EUCAST method and the Etest (ET_{DIR} and ET_{SD}) were similar ($P > 0.05$).

The essential agreement between ET_{DIR} and ET_{SD} of micafungin and anidulafungin was 98.3% and 100%, respectively, with few exceptions (Table 2). The essential agreement between ET_{DIR} and the EUCAST method was >97% for both micafungin and anidulafungin and 100% for micafungin against *Candida parapsilosis* and *Candida tropicalis* and for anidulafungin against *C. glabrata*.

The categorical agreement between ET_{DIR} and ET_{SD} was 100% for all species and

TABLE 2 Essential agreement and categorical agreement between the methods for micafungin and anidulafungin

Species	Agreement (% of isolates)							
	Essential[a]				Categorical			
	ET_{SD} vs ET_{DIR}[b]		EUCAST vs ET_{SD}/ET_{DIR}		EUCAST vs ET_{DIR}		*FKS* sequence vs ET_{DIR}/EUCAST	
	MYC[c]	AND[d]	MYC	AND	MYC	AND	MYC	AND
C. albicans	100	100	95.2/95.2	95.2/95.2	100	100	100	100
C. parapsilosis	100	95	100/100	90/95	100	100	100	100
C. tropicalis	100	100	100/100	95.6/95.6	100	91.3	100	100
C. glabrata	96.1	100	98/98.1	100/100	100	100	94.2	94.2
Overall	98.3	100	98.2/98.2	97.5/97.4	100	98.3	97.4	97.4

[a]Percentages of isolates in which the antifungal MIC differed ±2-log dilutions over the methods.
[b]ET_{SD}, Etest standard; ET_{DIR}, Etest direct.
[c]MYC, micafungin.
[d]AND, anidulafungin.

echinocandins. The categorical agreement between ET_{DIR} and the EUCAST method was 100% for micafungin and 98.3% for anidulafungin, where misclassifications were found in two *C. tropicalis* isolates (8.7% of major errors in *C. tropicalis*) in which the EUCAST method indicated resistance to micafungin but susceptibility to anidulafungin, whereas ET_{DIR} (and ET_{SD}) indicated resistance to both drugs (Table 3 and Table 2). The isolates harbored *FKS1* HS1 mutations (F641L and R647G) (Fig. 1). The categorical agreement between ET_{DIR} and the *FKS* sequence was 97.4% for both echinocandins (Table 2); the agreement was 100% for all species, with the exception of *C. glabrata* (94.2%), because of three isolates in which ET_{DIR} (and the EUCAST method) for both echinocandins indicated resistance but the *FKS1* and *FKS2* sequences were the wild types (overall 2.6% of major errors). Figure 2 shows the distributions of the micafungin and anidulafungin MICs obtained by ET_{DIR} and the EUCAST method (*C. parapsilosis* was excluded). An ET_{DIR} MIC of anidulafungin of ≥0.19 mg/liter and/or an MIC of micafungin of ≥0.064 mg/liter against *Candida albicans*, *C. tropicalis*, and *C. glabrata* effectively separated the phenotypically resistant isolates/*FKS* mutants from the susceptible isolates/*FKS* wild types (100% categorical agreement with combined gold standards for both agents).

Overall, the categorical agreement between the EUCAST method and growth on anidulafungin-containing plates was 62.6%. Major errors were found in all *C. albicans* and *C. tropicalis* echinocandin-susceptible isolates (the EUCAST method indicated susceptibility, but growth was visible on the plates [34.8%]). All *C. parapsilosis* isolates were able to grow on the plates. The best agreement was observed for *C. glabrata* (94.2%), with 100% of susceptible isolates not growing on the plates and 90.3% of the resistant isolates growing on the plates. Very major errors (the EUCAST method indicated resistance but there was no visible growth on the plates [2.6%]) were found in the three echinocandin-resistant *FKS* wild-type *C. glabrata* isolates.

DISCUSSION

Our study shows that performing the ET_{DIR} directly on positive blood cultures can speed up echinocandin susceptibility testing within 24 h of the detection of *Candida*. The results obtained by this rapid, easy, and inexpensive procedure mirrored those obtained by the EUCAST method and ET_{SD} and those obtained with the *FKS* gene sequence.

Current Infectious Diseases Society of America (IDSA) guidelines for the treatment of patients with candidemia recommend echinocandin susceptibility testing on isolates causing fungemia, particularly for patients previously exposed to echinocandins or infected by *C. glabrata* (5). Microdilution methods are preferred, although they require pure-cultured isolates, they are time consuming, and the results are not available until 48 to 72 h after diagnosis. Given that a delay in starting an appropriate antifungal treatment invariably leads to a poorer prognosis, the results of antifungal susceptibility must be anticipated where possible.

We previously showed that ET_{DIR} performed directly on positive blood cultures

TABLE 3 Micafungin and anidulafungin MICs against the 35 echinocandin-resistant *Candida* species isolates obtained using the EUCAST, ET_{SD}, and ET_{DIR} procedures

Species	*FKS* mutation	MIC (mg/liter)[a]					
		EUCAST		ET_{DIR}[b]		ET_{SD}[c]	
		MYC[d]	AND[e]	MYC	AND	MYC	AND
C. albicans	F641S (*FKS1* HS1)	1	0.25	>32	>32	>32	>32
C. tropicalis[f]	R647G (*FKS1* HS1)	0.25	0.064	0.38	0.19	0.38	0.19
C. tropicalis	S645F (*FKS1* HS1)	2	1	3	2	2	2
C. tropicalis[f]	F641L (*FKS1* HS1)	0.25	0.064	0.19	0.38	0.19	0.38
C. glabrata	Δ659 (*FKS2* HS1)	1	0.5	0.25	0.25	0.25	0.25
C. glabrata	Δ659 (*FKS2* HS1)	0.064	1	1.5	2	1.5	2
C. glabrata	Δ659 (*FKS2* HS1)	4	4	>32	>32	>32	>32
C. glabrata	Δ659 (*FKS2* HS1)	2	1	1	1.5	0.5	1
C. glabrata	Δ659 (*FKS2* HS1)	4	2	2	4	1.5	3
C. glabrata	Δ659 (*FKS2* HS1)	2	1	1	3	0.75	1
C. glabrata	Δ659 (*FKS2* HS1)	4	4	1.5	2	2	4
C. glabrata	Δ659 (*FKS2* HS1)	4	2	2	4	2	2
C. glabrata	Δ659 (*FKS2* HS1)	4	2	1	3	1	1
C. glabrata	Δ659 (*FKS2* HS1)	1	2	1.5	3	1	2
C. glabrata	Δ659 (*FKS2* HS1)	1	2	1.5	2	1.5	2
C. glabrata	Δ659 (*FKS2* HS1)	1	1	0.5	1.5	0.5	1
C. glabrata	Δ659 (*FKS2* HS1)	4	2	1	3	2	2
C. glabrata	Δ659 (*FKS2* HS1)	2	4	3	4	3	4
C. glabrata	S663P (*FKS2* HS1)	2	1	3	2	2	2
C. glabrata	S663P (*FKS2* HS1)	2	2	1	2	4	8
C. glabrata	S663P (*FKS2* HS1)	4	4	6	3	1	2
C. glabrata	S663P (*FKS2* HS1)	4	2	4	4	2	2
C. glabrata	S663P (*FKS2* HS1)	4	2	4	4	1	2
C. glabrata	S663P (*FKS2* HS1)	2	1	3	1.5	2	3
C. glabrata	S663P (*FKS2* HS1)	2	2	6	4	0.5	1
C. glabrata	W715L (*FKS2*)	2	2	2	4	2	2
C. glabrata	W715L (*FKS2*)	0.5	0.5	2	2	0.38	0.5
C. glabrata	W715L (*FKS2*)	4	2	8	1	4	2
C. glabrata	D666N (*FKS2* HS1)	0.064	0.25	0.125	0.5	0.125	0.5
C. glabrata	D666N (*FKS2* HS1)	0.064	0.5	0.064	0.38	0.094	0.38
C. glabrata	S663Y (*FKS2* HS1)	1	2	1	2	0.5	1.5
C. glabrata	E655A (*FKS2*)	0.25	0.5	0.125	1	0.125	0.38
C. glabrata[g]	Wild type	0.06	0.25	0.125	0.5	0.06	0.25
C. glabrata[g]	Wild type	0.06	0.25	0.19	0.38	0.125	0.38
C. glabrata[g]	Wild type	0.06	0.25	0.125	0.5	0.125	0.25

[a]EUCAST breakpoints used to classify the isolates as resistant: *C. albicans* (micafungin, >0.016; anidulafungin, >0.032); *C. glabrata* (micafungin, >0.032; anidulafungin, >0.064); *C. tropicalis* (micafungin [based on ECOFF], >0.06; anidulafungin, >0.032) (34, 37).
[b]ET_{DIR}, Etest direct.
[c]ET_{SD}, Etest standard.
[d]MYC, micafungin.
[e]AND, anidulafungin.
[f]*C. tropicalis* isolates showing resistance to micafungin but susceptibility to anidulafungin by the EUCAST method; both Etest procedures showed resistance to micafungin and anidulafungin.
[g]*C. glabrata* isolates showing phenotypic resistance to anidulafungin and micafungin but wild-type *FKS1* and *FKS2* genes.

showed good agreement with the CLSI M27-A3 procedure (2). The study proved useful for ruling out false resistance, which can facilitate antifungal de-escalation (for example, switching from echinocandins to fluconazole). As for the ability of ET_{DIR} to detect resistance, we demonstrated that it was reliable for caspofungin-resistant basidiomycete yeast or fluconazole-resistant non-*albicans Candida*. Unfortunately, we did not test fluconazole-resistant *C. albicans* isolates or echinocandin-resistant *Candida* species isolates. Our subsequent study demonstrated that ET_{DIR} was able to detect fluconazole-resistant *C. albicans* isolates (16). We conducted the present study with well-characterized echinocandin-resistant *Candida* species to complete the testing. We studied the agreement between ET_{DIR} and the EUCAST reference method for micafungin and anidulafungin. Caspofungin, the agent tested in our first paper (2), was not tested here owing to interlaboratory variability (18). For that reason, EUCAST does not provide caspofungin breakpoints.

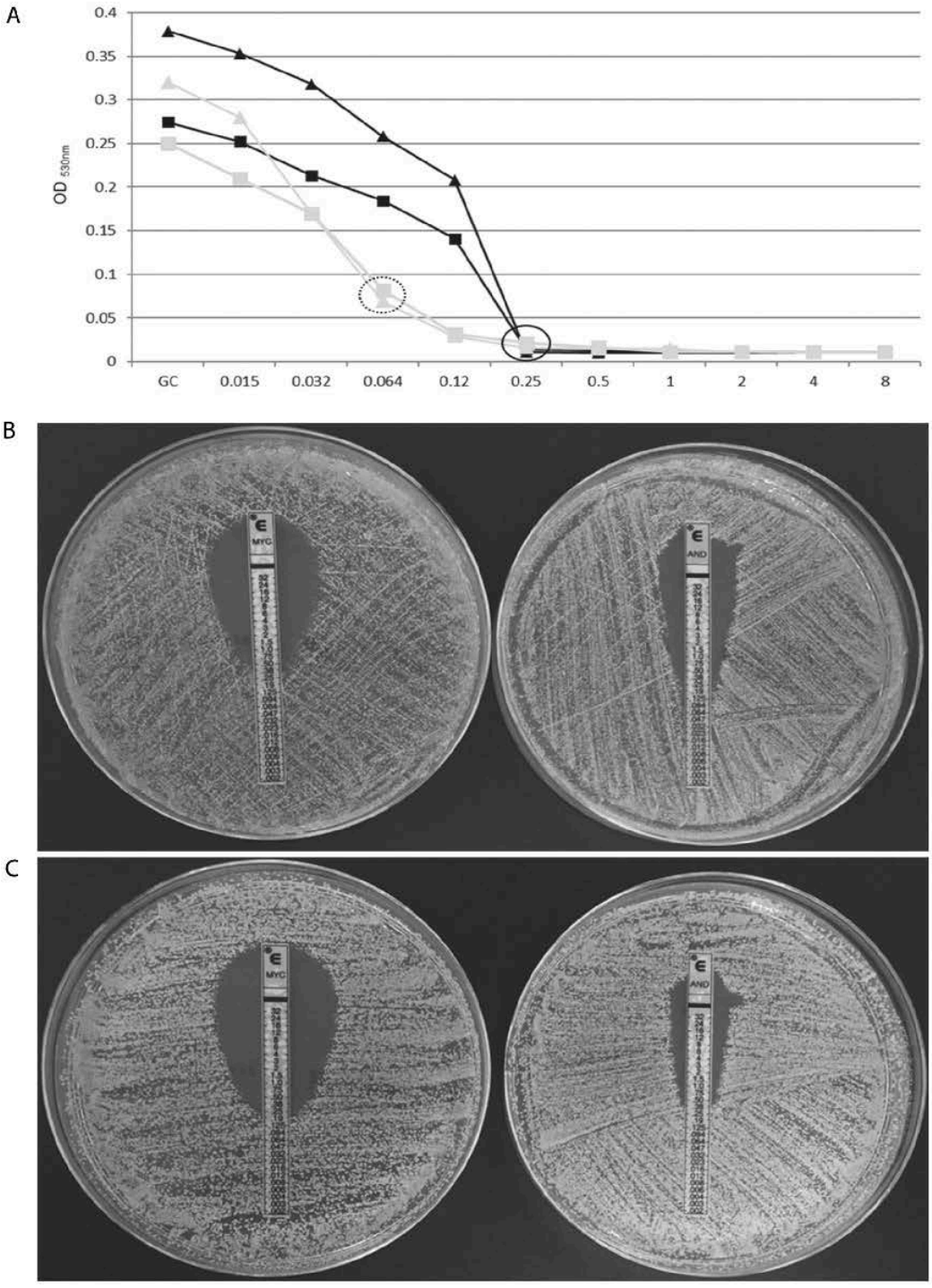

FIG 1 (A) Echinocandin growth inhibition curves obtained by the EUCAST method indicating resistance to micafungin (black) but susceptibility to anidulafungin (grey) for *C. tropicalis* isolates with R647G (triangles) or F641L (squares) mutations. Black circle indicates micafungin MIC; dotted circle indicates anidulafungin MIC; GC, growth control. ET_{DIR} of two *C. tropicalis* isolates with R647G (B) and F641L (C) mutations indicating resistance to both echinocandins. MYC, micafungin; AND, anidulafungin.

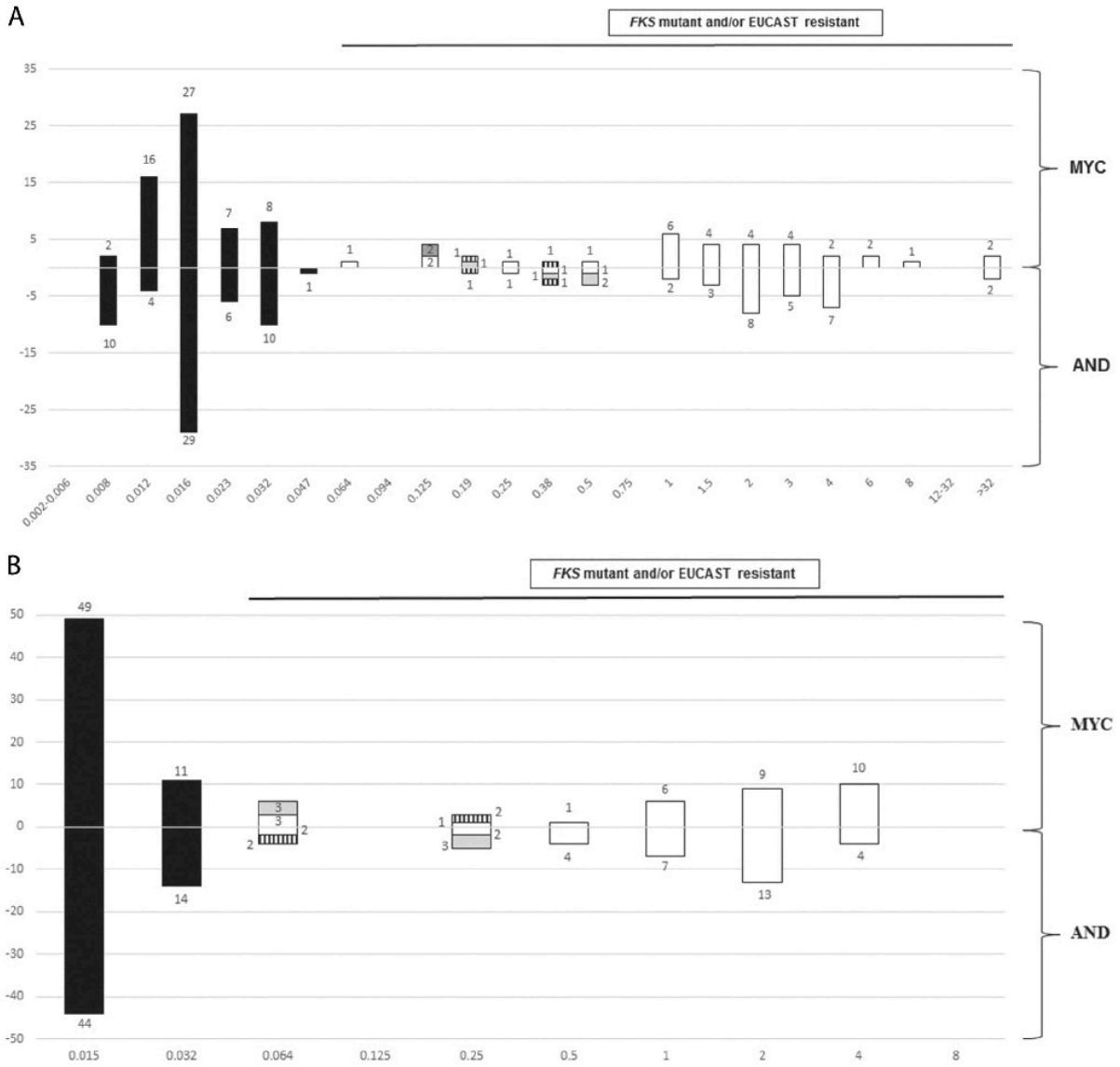

FIG 2 Distribution of the micafungin (MYC) and anidulafungin (AND) MICs obtained by ET_{DIR} (A) and the EUCAST method (B) against 95 isolates (*C. parapsilosis* isolates were excluded). Black bars, *FKS* wild-type isolates classified as susceptible by the EUCAST method; grey bars, *FKS* wild-type *C. glabrata* isolates classified as resistant by the EUCAST method; hatched bars, *FKS* mutant *C. tropicalis* isolates classified as anidulafungin susceptible and micafungin resistant by the EUCAST method; white bars, *FKS* mutant isolates classified as resistant by the EUCAST method.

We found very high essential and categorical agreement between the ET_{DIR}, ET_{SD}, and EUCAST methods for echinocandin susceptibility testing against *Candida* spp. No very major errors (false susceptibility) were detected for any of the isolates tested. However, a few major errors were found for anidulafungin in two *C. tropicalis* isolates with *FKS1* mutations that were classified as anidulafungin-susceptible and micafungin-resistant by the EUCAST method and as resistant by ET_{DIR}. Previous studies reported *C. albicans* and *Candida kefyr* isolates with the *FKS1* mutations R647G and P649H and the above-mentioned phenotype using CLSI methods (19, 20), thus suggesting that the amino acids R647 and P649 are key for glucan synthase inhibition by micafungin. Another study reported a *C. tropicalis* isolate harboring the F641L *FKS1* mutation with

dose-dependent susceptibilities to both micafungin and anidulafungin, again, by using CLSI methods (21). Since EUCAST does not yet have breakpoints for micafungin against *C. tropicalis*, our isolates had to be classified using epidemiologic cutoff values (ECOFFs). However, our and other previously reported observations for *C. glabrata* (22–25) suggest that the presence of *FKS* mutations itself is not sufficient to predict the pattern of resistance to anidulafungin or micafungin. Therefore, we decided to test anidulafungin and micafungin in parallel. On the basis of our results, micafungin may be a good surrogate marker of echinocandin resistance in *C. albicans* and *C. tropicalis* isolates.

To improve the potential of ET_{DIR} for the detection of both resistant and *FKS* non-wild-type isolates, we calculated the categorical agreement using the *FKS* sequence as the gold standard. The categorical agreement between the *FKS* sequence and ET_{DIR} was very high, thus showing the ability of ET_{DIR} to discriminate between *FKS* mutants and wild types in most cases. However, three major errors were observed in three unusual *C. glabrata* isolates that showed very high anidulafungin and micafungin MICs by the EUCAST method and Etest but wild-type *FKS1* and *FKS2* sequences. We cannot rule out alternative mechanisms of resistance (e.g., efflux pumps), although the ET_{DIR} classified them correctly, in agreement with the EUCAST method, as resistant, suggesting the ability of this procedure to detect wild-type *FKS*/resistant isolates. Future studies on these isolates are required. To separate the *FKS* wild-type/susceptible isolates from *FKS* mutant/resistant isolates, we suggest the following cutoffs for ET_{DIR}: anidulafungin, ≥0.19 mg/liter; and/or micafungin, ≥0.06 mg/liter.

Recent studies have proved that azole-containing plates are useful when screening for the presence of resistance in *Aspergillus* (26). To determine whether this procedure would be useful for the screening of echinocandin resistance in *Candida*, we decided to use anidulafungin-containing plates at 2 mg/liter on the basis of our previous study, which reports the anidulafungin mutant prevention concentration (27). However, we found poor agreement between this method and the EUCAST method owing to the high percentages of false resistance in *C. albicans* and *C. tropicalis*. A paradoxical effect is common to both species (28, 29). Owing to the lack of echinocandin-resistant *C. parapsilosis* isolates tested and the fact that 100% of the intermediate isolates grew on the plates, this procedure cannot be recommended for *C. parapsilosis*, *C. albicans*, or *C. tropicalis*.

Our main limitation is the low number of *C. albicans* and *C. tropicalis FKS* mutant isolates. However, the acquisition of echinocandin resistance involves *C. glabrata* to a greater extent than other *Candida* spp. (11, 12), and the number of *C. glabrata* mutant isolates tested was moderately high. Furthermore, the positive results reported here reinforce our previous observation of basidiomycete yeast being correctly classified as caspofungin resistant (2).

We conclude that the ET_{DIR} for micafungin and anidulafungin is a reliable and fast procedure when screening for the presence of echinocandin resistance in *Candida* species causing candidemia and can be easily implemented in the routine of the microbiology laboratory.

MATERIALS AND METHODS

Samples. We prospectively collected 80 positive blood cultures (Bactec-FX system, Becton-Dickinson, Cockeysville, MD, USA) to screen for echinocandin-susceptible *Candida* spp. (*C. albicans*, $n = 20$; *C. tropicalis*, $n = 20$; *C. glabrata*, $n = 20$) and echinocandin-intermediate *C. parapsilosis* ($n = 20$) from patients with candidemia admitted to Gregorio Marañón Hospital (Madrid, Spain) between 2010 and 2013. A total of 1 to 2 ml of broth from each bottle was stored at −70°C. Additionally, 0.5-ml (0.5 McFarland) suspensions of 35 echinocandin-resistant *Candida* species isolates obtained in previous studies (2, 27, 30–32) were artificially spiked in nonfungemic/bacteremic Bactec bottles until they were flagged as positive (Table 3). All 115 isolates were identified by amplification and sequencing of the ITS1-5.8S-ITS2 regions (33).

EUCAST antifungal susceptibility testing and ET_{SD}. All isolates were tested for susceptibility to micafungin (Astellas Pharma, Inc., Tokyo, Japan) and anidulafungin (Pfizer Pharmaceutical Group, New York, NY, USA) according to the EUCAST E.DEF 7.3.1 microdilution procedure (34–36). The echinocandin concentrations ranged from 0.015 to 8 mg/liter. Inoculated plates were incubated for 24 h at 35°C.

ET_{SD} of micafungin and anidulafungin was performed on the isolates according to the manufacturer's instructions. Briefly, the suspensions were prepared, adjusted to 0.5 McFarland, and stroked on RPMI 1640 agar plates supplemented with 2% glucose (bioMérieux, Marcy-l'Etoile, France). The strips were placed on the agar surfaces of the plates, which were then incubated at 35°C for 24 h. The MIC was set when the fungal elliptic growth intersected the plastic strip.

Antifungal susceptibility testing performed directly on blood samples using ET_{DIR} and anidulafungin-containing agar plates. A total of 2 to 4 drops of the broth medium (stored broth from the 80 isolates preincubated overnight at 37°C and positive flagged bottles of the 35 echinocandin-resistant isolates) were stroked on RPMI 1640 agar plates on which Etest strips of micafungin and anidulafungin had been placed; 2 to 4 drops of the broth medium were also stroked on Sabouraud agar plates containing 2 mg/liter of anidulafungin. The plates were incubated at 35°C for 24 h.

Data analysis. The geometric mean (GM) MICs of micafungin and anidulafungin against the isolates obtained by the three methods were calculated and compared using the *t* test, with a *P* value of <0.05 considered statistically significant. MICs obtained using EUCAST 7.3.1 were considered the gold standard and were compared with those obtained by ET_{DIR} and ET_{SD} to calculate the essential agreement between the methods (percentage of isolates in which MIC differed by $\pm$2-log dilutions over the reference method). All isolates were classified as resistant or susceptible according to the clinical breakpoints proposed by EUCAST for any of the three methods (37). Given the lack of clinical breakpoints for micafungin against *C. tropicalis*, we tentatively considered isolates showing an MIC above the ECOFF ($>$0.06 mg/liter) to be resistant in order to avoid the term "non-wild-type," which is used exclusively for *FKS* mutants (34). The procedures were in categorical agreement when the results were in the same susceptibility category (2) based on two gold standards: the EUCAST method and *FKS* sequence (regardless of the MIC). The anidulafungin-containing plate-screening procedure was in categorical agreement with the EUCAST method when resistant isolates or *FKS* mutants were able to grow on the plates and susceptible isolates or *FKS* wild types were unable to grow visibly on the plates. Errors were categorized as very major (agar diffusion methods indicated susceptibility and the EUCAST method/*FKS* sequences indicated resistance or mutations) or major (agar diffusion methods indicated resistance and the EUCAST method/*FKS* sequence indicated susceptibility or wild type).

Ethical considerations. This study was approved by the ethics committee of Hospital Gregorio Marañón (CEIC-A1, study no. 208/16).

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6.3.2. Artículo 7: Detection of echinocandin-resistant *Candida glabrata* in blood cultures spiked with different percentages of *FKS2* mutants.

En este estudio se comparó el método EUCAST con el Etest® y las placas de agar con anidulafungina realizados directamente sobre hemocultivos positivos para detectar rápidamente resistencia a las equinocandinas en presencia de cepas de *C. glabrata* tanto sensibles como resistentes coexistiendo en diferentes proporciones.

Se estudiaron diez parejas de *C. glabrata* (cepa parental sensible y cepa isogénica resistente a las candinas). Para cada pareja se prepararon tres inóculos (1-5 $x10^3$, 1-5 $x10^2$ y 10-50 UFC/mL) que abarcaban suspensiones con diferentes proporciones de la cepa sensible/resistente (9/1, 5/5 y 1/9). Las nueve suspensiones (por pareja) se inocularon en botellas de hemocultivos negativas que se re-incubaron hasta su positividad. Posteriormente se aplicaron 3-6 gotas del medio del hemocultivo en placas de Sabouraud para realizar el método de EUCAST, en placas de RPMI para realizar el Etest® directo (ET_{DIR}) y en placas de agar con anidulafungina (2 mg/L).

El método de EUCAST detectó resistencia a las equinocandinas cuando las botellas contenían cepas sensibles/resistentes en las proporciones de 5/5 y 1/9. En cambio, las suspensiones con una proporción 9/1 en tres parejas resultaron sensibles a las equinocandinas. Sin embargo, el ET_{DIR} detectó resistencia a micafungina (CMI ≥0,064 mg/L) y a anidulafungina (CMI ≥0,125 mg/L) en todas las parejas, independientemente de la proporción y del inóculo utilizado. Las placas con anidulafungina permitieron el crecimiento de todas las cepas tras 48 horas de incubación.

Se concluyó que el método del Etest® realizado directamente sobre hemocultivos positivos fue el método más rápido y fiable para detectar resistencia a las equinocandinas en *C. glabrata*, incluso cuando los aislados resistentes están infrarrepresentados.

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SUSCEPTIBILITY

Detection of Echinocandin-Resistant *Candida glabrata* in Blood Cultures Spiked with Different Percentages of *FKS2* Mutants

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ABSTRACT Infections caused by the coexistence of *Candida glabrata* echinocandin-resistant and echinocandin-susceptible cells may be possible, and the detection of *FKS* mutants when the proportions of *FKS* mutants are underrepresented poses a problem. We assessed the role of EUCAST and methods directly performed on positive blood cultures—Etest (ET_{DIR}) and anidulafungin-containing agar plate assays—for detecting resistance in *C. glabrata* isolates containing different amounts of echinocandin-susceptible and -resistant *Candida glabrata* isolates. We studied 10 pairs of *C. glabrata* isolates involving parental echinocandin-susceptible isolates and isogenic echinocandin-resistant *FKS* mutant isolates. Three inocula per pair (1×10^3 to 5×10^3, 1×10^2 to 5×10^2, and 10 to 50 CFU/ml) spanning suspensions with different amounts of susceptible/resistant isolates (9/1, 5/5, and 1/9 isolates for each the three inocula) were prepared. The suspensions were spiked in Bactec bottles and incubated until they were positive, and the three methods were compared. The EUCAST method showed echinocandin resistance when the bottles were spiked with resistant isolates at 5/5 and 1/9 proportions; the results for the suspensions with a 9/1 proportion of resistant isolates were susceptible for three pairs. We observed with the ET_{DIR} resistance to both echinocandins in all pairs (resistance to micafungin and anidulafungin; MICs, ≥0.064 mg/liter and ≥0.125 mg/liter, respectively) and a double ring of growth inhibition in two pairs. The anidulafungin-containing plates showed fungal growth in the 90 spiked blood cultures at 48 h. Testing of echinocandin susceptibility with the ET_{DIR} directly on the positive blood culture bottles is a reliable and rapid method to detect echinocandin resistance in *C. glabrata*. On the other hand, resistance can be missed with the EUCAST method when resistant isolates are underrepresented.

KEYWORDS *Candida glabrata*, EUCAST procedure, echinocandins, Etest, resistance

The incidence of invasive fungal infections is increasing, and mortality rises when the initiation of appropriate antifungal therapy is delayed (1–3). *Candida glabrata* is one of the main causes of invasive candidiasis, and its occurrence is growing (4, 5). Among the most important factors associated with invasive *C. glabrata* infections are the use of broad-spectrum antibiotics, catheters, and parenteral nutrition; the presence of immunosuppression; the disruption of mucosal barriers; and chemotherapy/radiotherapy (6).

Echinocandin resistance in *C. glabrata* poses a problem for the management of patients due to its intrinsic low level of susceptibility to azoles and the poor prognosis for patients infected by echinocandin-resistant isolates (4, 5). The risk factors for developing echinocandin-resistant *C. glabrata* candidemia are previous echinocandin

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TABLE 1 Micafungin and anidulafungin MICs against the isolates from bottles spiked with the different tested inocula and proportions

Inoculum (CFU/ml)	Proportion	EUCAST micafungin/anidulafungin MIC (mg/liter) for the following pair[a]:									
		1	2	3	4	5	6	7	8	9	10
1×10^3–5×10^3	9S/1R	1/2	2/1	4/2	0.25/0.5	0.064/0.125	**0.015/0.064**	**0.015/0.064**	0.5/2	**0.015/0.064**	0.125/0.25
	5S/5R	2/2	2/1	4/2	0.25/0.5	0.064/0.25	1/2	1/1	1/2	0.5/1	4/2
	1S/9R	1/2	1/0.5	4/2	0.5/1	0.064/0.25	1/1	1/2	1/2	0.5/1	4/2
1×10^2–5×10^2	9S/1R	1/0.5	0.064/0.125	2/2	0.25/0.5	0.064/0.125	**0.015/0.064**	**0.015/0.064**	0.5/1	**0.015/0.064**	0.25/0.25
	5S/5R	0.5/0.5	0.5/0.25	4/2	0.25/0.5	0.064/0.125	1/1	2/1	0.5/2	0.5/0.5	4/2
	1S/9R	0.25/0.125	0.25/0.125	4/2	0.25/0.5	0.064/0.25	1/2	1/2	1/2	0.5/1	4/2
10–50	9S/1R	1/0.5	0.25/0.125	1/0.5	0.25/0.5	0.064/0.125	**0.015/0.064**	**0.015/0.064**	1/2	**0.032/0.064**	0.125/0.25
	5S/5R	1/0.5	0.5/0.125	1/0.5	0.25/0.5	0.064/0.125	1/0.5	0.5/0.25	1/2	0.5/0.5	4/2
	1S/9R	2/1	2/1	4/2	0.5/0.5	0.064/0.25	1/2	2/1	0.5/2	0.5/0.5	4/2

[a]Bold numbers indicate EUCAST MICs for echinocandin susceptibility for the tested isolates after preparing the inoculum from slime.

exposure, solid organ transplantation, recent gastrointestinal surgery or a recent gastrointestinal disorder, and multiple episodes of *C. glabrata* bloodstream infections (4, 7). Moreover, recent studies have reported that the abdominal cavity and mucosal surfaces may serve as reservoirs for resistant isolates (8, 9). Echinocandins are indicated to be the first line of treatment in cases of invasive candidiasis (10), a recommendation supported by the low rate of echinocandin resistance (1, 11, 12). However, some studies have provided alerts on the increased rates of echinocandin resistance in *C. glabrata* strains causing infection in some geographic areas (4, 5). Echinocandin resistance in *C. glabrata* is associated with the presence of mutations in hot spots of the *FKS1* and *FKS2* genes (5).

The rapid detection of echinocandin resistance in *C. glabrata* in blood samples can contribute to the improvement of patient care. Molecular detection of resistance would speed up the results, although to date these techniques are pending on validation for their use with blood samples (5, 13). In a previous study, we showed that the Etest directly performed on positive blood cultures (ET_{DIR}) is a reliable procedure to rapidly detect fluconazole- and echinocandin-resistant isolates (14–16). Moreover, anidulafungin-containing plates were useful to screen for the presence of echinocandin-resistant *C. glabrata* isolates directly from positive blood cultures (16).

Data on the antifungal susceptibility obtained using standardized testing procedures, such as the CLSI or EUCAST procedures, are mainly obtained from isolates recovered from automated blood culture systems, such as the Bactec FX system (Becton, Dickinson, Cockeysville, MD, USA) (17, 18). The scenario in which infections are caused by the coexistence of echinocandin-resistant cells and echinocandin-susceptible ones may be possible. In situations in which the proportion of *C. glabrata FKS* mutants in culture is underrepresented in comparison to the proportion of wild-type isolates, the reliability of detection of *C. glabrata FKS* mutants using standard methods and rapid methods (anidulafungin-containing agar plate assays or ET_{DIR}) is unknown.

In this study, we aimed to examine the accuracy of the EUCAST EDef 7.3.1 standard procedure and the rapid techniques (ET_{DIR} and anidulafungin-containing agar plates) for assessing susceptibility to echinocandin antifungals in *C. glabrata* isolates using inocula with different proportions of echinocandin-susceptible and echinocandin-resistant *C. glabrata* isolates.

RESULTS

Antifungal susceptibility of isolates spiked in bottles following the EUCAST standard procedure. Ninety bottles were spiked with the nine possible combinations of inocula and different proportions of susceptible/resistant isolates. The antifungal susceptibility of the isolates was performed from the slime on the plates and is shown in Table 1. The isolates in cultures from bottles spiked with suspensions containing

TABLE 2 Number of individual colonies obtained from each culture from the 90 spiked bottles[a]

Inoculum (CFU/ml)	Proportion	No. of colonies for the following pair:																			
		1		2		3		4		5		6		7		8		9		10	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R	S	R
1×10^3–5×10^3 ($n = 266$ colonies)	9S/1R	7	0	4	2	10	0	4	4	5	2	5	3	5	4	9	0	8	1	7	2
	5S/5R	7	3	5	5	4	6	6	4	5	4	4	5	6	4	6	4	4	4	6	3
	1S/9R	2	6	3	7	2	5	2	8	3	5	2	8	4	5	0	8	4	6	3	6
1×10^2–5×10^2 ($n = 253$ colonies)	9S/1R	5	1	6	3	4	1	8	2	6	2	4	5	6	4	8	2	8	1	6	2
	5S/5R	2	3	6	4	0	6	7	3	6	3	4	5	4	3	7	2	6	3	5	4
	1S/9R	0	6	2	8	0	6	5	5	5	4	2	7	4	6	1	9	3	5	3	5
10–50 ($n = 233$ colonies)	9S/1R	6	3	4	2	4	2	5	1	6	3	5	4	4	4	7	1	8	1	7	2
	5S/5R	4	4	3	2	0	6	5	3	4	5	4	5	5	4	4	4	4	4	7	2
	1S/9R	0	6	3	5	0	4	4	6	3	5	3	5	2	5	2	5	3	5	6	3

[a]Colonies were classified as susceptible or resistant to both echinocandins according to the EUCAST clinical breakpoints. S, echinocandin-susceptible colonies; R, echinocandin-resistant colonies.

susceptible/resistant isolates in proportions of 5/5 and 1/9 were phenotypically resistant to both micafungin and anidulafungin. On the other hand, the isolates in cultures from 3 out of the 10 bottles (pairs 6, 7, and 9) spiked with suspensions of susceptible/resistant isolates in proportions of 9/1 were susceptible to both echinocandins (Table 1).

Seven hundred fifty-two individual colonies from the 90 bottles ($n = 266$, $n = 253$, and $n = 233$ colonies from the 10^3-, 10^2-, and 10-CFU/ml inocula, respectively) were tested and determined to be susceptible ($n = 393$) or resistant ($n = 359$) to both echinocandins (Table 2). Overall differences in the number/percentage of echinocandin-resistant colonies ($n = 124/46.6\%$, $n = 120/47.4\%$, and $n = 115/49.4\%$ from the 10^3-, 10^2-, and 10-CFU/ml inocula, respectively) did not reach statistical significance ($P > 0.05$). However, the higher that the proportion of resistant isolates in the suspension used to spike the bottles was, the higher that the proportion of resistant colonies counted on the plates was, regardless of the inoculum used ($P < 0.05$) (see Fig. S2 in the supplemental material). This was consistently observed for every tested pair (Table 2). Colonies in cultures from bottles spiked with suspensions with susceptible/resistant isolates in 5/5 and 1/9 proportions were either susceptible or resistant to echinocandins. However, resistant colonies were missing from three pairs of cultures from bottles spiked with suspensions with susceptible/resistant isolates in a 9/1 proportion (pairs 1, 3, and 8; Table 2).

Antifungal susceptibility testing using ET_{DIR}. Ninety ET_{DIR} tests to detect anidulafungin and micafungin susceptibility were performed. Using the breakpoints of EUCAST, ET_{DIR} classified the isolates from the 90 bottles as resistant to both echinocandins. A wide distribution of MICs was observed, regardless of the proportion of susceptible/resistant isolates spiked in the blood culture (Fig. 1A). Conversely, the type of mutation was of great relevance regarding the MICs obtained by ET_{DIR}; certain mutations leading to high MICs for both echinocandins by the EUCAST method resulted in elevated ET_{DIR} MICs (Fig. 1B). The setting of the MIC was easy in most cases, but the ET_{DIR} showed the presence a double ring of growth inhibition for pairs 7 and 8. The thickness of the inner halo (probably representing the resistant isolate) increased with higher proportions of the resistant isolate in the suspension used to spike the blood culture; inner halo growth was taken into account to set the MIC (Fig. 2).

Screening of resistance on anidulafungin-containing agar plates. Two fungal growth patterns were seen for the 90 spiked blood cultures in the plates incubated for 24 to 48 h. Slime-like growth was detected at 24 h of incubation, whereas single colonies were noticed in pairs 4, 5, and 9, which turned positive only when the incubation was extended to 48 h. Furthermore, the isolates producing single colonies were from blood cultures spiked with isogenic isolates with lower MICs of anidulafun-

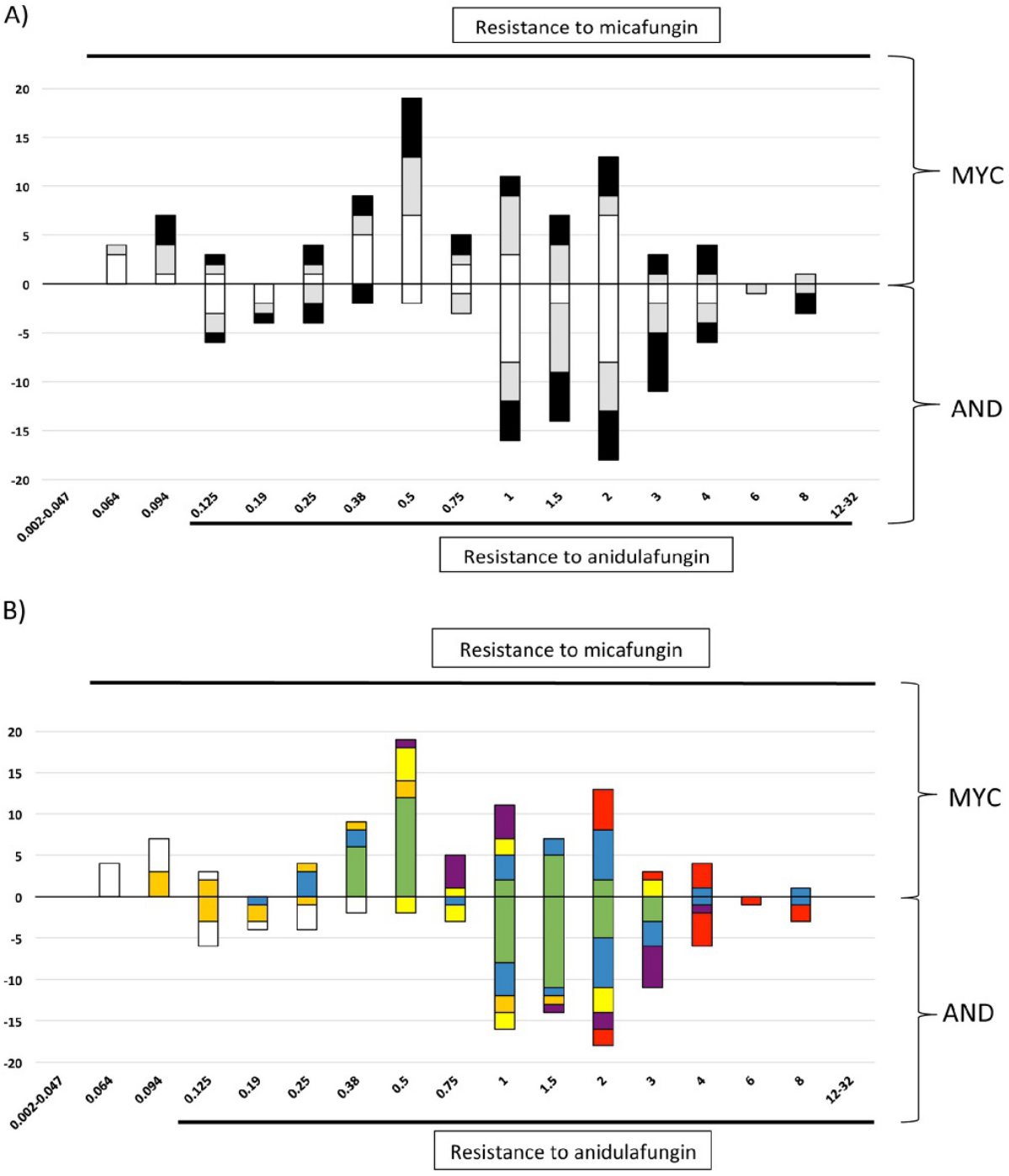

FIG 1 Distribution of micafungin and anidulafungin MICs obtained using ET_{DIR} of cultures from the 90 spiked bottles. (A) Micafungin (MYC) and anidulafungin (AND) MICs for isolates obtained from blood cultures spiked with suspensions containing different proportions of susceptible/resistant isolates (white bars, 9/1; gray bars, 5/5; black bars, 1/9). (B) The results for isolates with different *FKS2* mutations, including the *FKS* wild-type isolate classified as resistant by the EUCAST method, are shown. White bars, *FKS* wild-type isolate classified as resistant by the EUCAST method; orange bars, isolate with the *FKS2* E655A mutation; blue bars, isolate with the *FKS2* S663P mutation; green bars, isolate with the *FKS2* ΔF658 deletion; yellow bars, isolate with the *FKS2* W715L mutation; purple bars, isolate with the *FKS2* S663Y mutation; red bars, isolate with the *FKS2* D666N mutation.

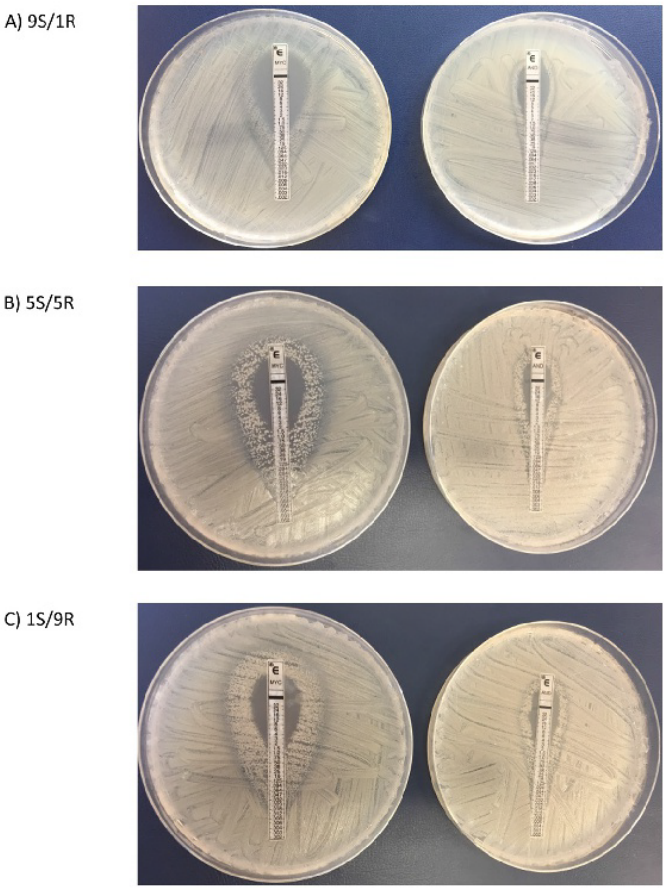

FIG 2 ET_{DIR} of micafungin and anidulafungin showing a double ring of growth inhibition in the 1×10^3- to 5×10^3-CFU/ml inoculum in suspensions containing different proportions of susceptible/resistant isolates: 9/1 (A) 5/5 (B), or 1/9 (C). MYC, micafungin; AND, anidulafungin.

gin and micafungin by the EUCAST method (Table 3). As mentioned above for ET_{DIR}, the results were not affected by the inoculum.

DISCUSSION

To the best of our knowledge, in this study we detected for the first time, using a Bactec FX automated blood culture system, echinocandin-resistant *C. glabrata* isolates present in low proportions, regardless of the type of *FKS2* gene mutation or echinocandin MIC. ET_{DIR} and assays with anidulafungin-containing agar plates performed directly with spiked positive blood cultures proved to be reliable procedures to detect echinocandin resistance in all the tested scenarios.

Current Infectious Diseases Society of America (IDSA) guidelines recommend echinocandin susceptibility testing on isolates causing fungemia, particularly in patients who had previously been exposed to echinocandins or infected by *C. glabrata* (10). The screening for echinocandin-resistant *C. glabrata* is a must, given the emergence of resistance in some institutions (4, 19, 20). The reasons for the differences in the rates of echinocandin resistance between institutions is unclear. These may be due to conditions of the blood culture systems that prevent resistant isolates from thriving or missed resistance detection when standard antifungal susceptibility testing methods, such as the EUCAST method, are used. Different proportions of susceptible/resistant isolates were spiked into the blood cultures. Thus, we performed antifungal suscepti-

TABLE 3 Micafungin and anidulafungin MICs for the isolates in each used pair to prepare the spiked suspensions in Bactec bottles and *FKS2* gene sequence of the tested isolates[a]

Pair	Isolate	EUCAST MYC/AND MIC (mg/liter)	*FKS2* gene sequence
1	Parental	0.015/0.032	WT
	Isogenic	4/2	ΔF658
2	Parental	0.015/0.032	WT
	Isogenic	4/2	ΔF658
3	Parental	0.015/0.032	WT
	Isogenic	4/2	S663P
4	Parental	0.015/0.032	WT
	Isogenic	0.25/0.5	E655A
5	Parental	0.015/0.032	WT
	Isogenic	0.064/0.25	WT
6	Parental	0.015/0.015	WT
	Isogenic	0.5/0.5	W715L
7	Parental	0.015/0.015	WT
	Isogenic	2/1	ΔF658
8	Parental	0.015/0.064	WT
	Isogenic	1/2	S663Y
9	Parental	0.015/0.032	WT
	Isogenic	0.064/0.5	D666N
10	Parental	0.015/0.032	WT
	Isogenic	2/1	S663P

[a]MYC, micafungin; AND, anidulafungin. Parental isolates were phenotypically echinocandin susceptible, and isogenic ones were phenotypically echinocandin resistant. Pairs 1 to 8 came from a previous study and involved susceptible isolates from blood samples exposed *in vitro* to either micafungin or anidulafungin and the corresponding resistant ones generated (27, 28). Pairs 9 and 10 originated in two patients with candidemia who developed concomitant echinocandin-resistant endocarditis. The parental and isogenic isolates in each pair proved to be genotypically identical. WT, wild type.

bility testing by preparing different inocula for the EUCAST method. When we tested a loopful from the slime, resistance was missed in 3 out of the 10 pairs with the lowest proportion of the resistant isolate. Not being able to detect resistance by the EUCAST method was not related to a *FKS2* mutation or to the MIC (Table 3). Likewise, resistance was also missed in blood cultures with the lowest proportion of resistant isolates after picking up single colonies from the plates in three pairs. This implies that the preparation of inoculum suspensions following the EUCAST EDef 7.3.1 method (21) (selecting 4 to 5 colonies from the plate) does not ensure the detection of resistance, as shown by pairs 1, 3 and 8, from which only susceptible colonies were obtained from the bottles spiked with the lowest proportion of resistant isolates (Table 2).

Since the EUCAST procedure does not ensure the detection of resistant isolates, we studied alternative methods, such as ET_{DIR} and assays with anidulafungin-containing plates. We had previously shown that ET_{DIR} performed directly with positive blood cultures allowed detection of resistance to fluconazole and echinocandins (14–16). Furthermore, we studied ET_{DIR} using cultures from bottles spiked with different proportions of echinocandin-susceptible/echinocandin-resistant *C. glabrata* isolates. ET_{DIR} showed micafungin and anidulafungin MICs of ≥0.064 mg/liter and ≥0.125 mg/liter, respectively; the MIC values depended on the type of *FKS2* mutation rather than on the proportion of resistant isolates and the inoculum spiked in the bottles. We did not spike the bottles with inocula containing only susceptible isolate in the pairs, because a previous study carried out by our group showed MICs of anidulafungin and micafungin of ≤0.047 mg/liter against the same isolates by ET_{DIR} (16). A double ring of growth inhibition was observed in some cases with ET_{DIR}; this phenomenon has previously been reported in other species, such as *Candida lusitaniae* with amphotericin B and *Aspergillus fumigatus* with caspofungin (22, 23). The wider that the inner halo is, the higher that the proportion of spiked resistant isolates is (Fig. 2). These results suggest that ET_{DIR} can rapidly (24 h) determine the presence of heteroresistance in the blood cultures, which can be missed using the EUCAST standard procedure.

The assay with antifungal-containing plates, an inexpensive and easy procedure to

rule out the presence of resistance, has recently been tested to screen antifungal resistance in *Candida* and *Aspergillus* (16, 24). In this study, we found that all cultures from anidulafungin-containing plates were positive, regardless of the proportion of resistant isolates or the inoculum used. However, the two detected growth patterns mirrored the MICs of the isolates: isolates with high MICs were easily detected after 24 h of incubation, whereas the other isolates, including the *FKS* wild-type, phenotypically resistant isolate, required up to 48 h of incubation. Likewise, in our previous study we showed that phenotypically susceptible isolates failed to grow on the plates (16).

The medium number of *Candida* spp. circulating in the bloodstream has been estimated to be ≤1 CFU/ml (range, 0.1 and >1,000 CFU/ml), and the number for *C. glabrata* is lower than that for other species (25). Our experimental conditions simulated real-life candidemia (assuming that 10 ml of blood from venipuncture was inoculated in the bottles and that the lowest inoculum spiked [10 to 50 CFU/ml] mimicked a load of 1 to 5 CFU/ml circulating in the blood). Given that the inoculum did not seem to have a great impact on the results, our experimental conditions can be extrapolated to clinical samples.

There are certain limitations in this study. First, we studied only *C. glabrata* isolates; however, the emergence of resistance to echinocandins and/or to multiple antifungals mainly affects this species (4, 19, 20). Second, not all *C. glabrata FKS1* and *FKS2* gene mutations have been studied, although the most commonly reported substitution, S663, was included among the six mutations tested in this study (5). Third, studies should be carried out with automatic systems other than the Bactec system. Fourth, the reliability of our procedure for the detection of mutants in cases of candidemia episodes caused by *Candida* blood loads below 1 CFU/ml is unknown. Finally, although the procedure worked well in our laboratory, future interlaboratory studies to validate the role of ET_{DIR} are warranted.

In conclusion, the Bactec automatic system allows the detection of echinocandin-resistant *C. glabrata* isolates from blood cultures. However, when resistant isolates are underrepresented, their detection can be missed with the EUCAST standard procedure. ET_{DIR} is a reliable and a rapid method to detect resistance to micafungin and anidulafungin, ensuring detection in potential situations of increasing echinocandin resistance.

MATERIALS AND METHODS

Isolates. We studied 10 pairs of molecularly identified *C. glabrata* isolates (26) involving parental echinocandin-susceptible isolates causing candidemia and isogenic echinocandin-resistant ones either generated *in vitro* ($n = 8$) (27, 28) or recovered from the heart valves of patients with concomitant endocarditis ($n = 2$). Microsatellite markers showed that the parental and isogenic isolates had the same genotype (29). The characteristics of the isolates are shown in Table 3.

Inocula used to spike blood culture bottles. McFarland 0.5 suspensions (corresponding to 1×10^6 to 5×10^6 CFU/ml) of each pair of susceptible and resistant isolates were prepared. The suspensions were diluted to 1×10^3 to 5×10^3, 1×10^2 to 5×10^2, and 10 to 50 CFU/ml. Finally, different proportions of susceptible/resistant isolates (9/1, 5/5, and 1/9) for each pair of each of the three tested inocula were prepared. The concentrations of the inocula and the proportions were confirmed through colony counting on Sabouraud dextrose agar plates (data not shown). Cultures from bottles previously inoculated with blood from patients that remained negative after 7 days of incubation were subsequently used for the experiments. The bottles were reincubated at 35°C under continuous agitation in a Bactec FX system until they were flagged as positive (range, 23.5 h to 65.5 h). One milliliter of each suspension was spiked in nonfungemic/bacteremic Bactec bottles (Bactec Plus Aerobic/F; Becton, Dickinson, Cockeysville, MD, USA) (9 bottles per pair) (see Fig. S1 in the supplemental material).

Antifungal susceptibility testing and screening for resistance. Antifungal susceptibility was determined following the EUCAST standard procedure and procedures performed directly on blood cultures (ET_{DIR} and resistance screening on anidulafungin-containing agar plates).

Five to 6 drops of the broth medium from the bottles flagged as positive were stroked onto Sabouraud dextrose agar plates, and the plates were incubated at 35°C for 24 h. A loopful of the slime growth was collected and suspended in water to examine susceptibility to micafungin and anidulafungin per the EUCAST EDef 7.3.1 method (21). Isolates were considered resistant to micafungin or anidulafungin when the MICs were above 0.032 mg/liter and 0.064 mg/liter, respectively. Additionally, to assess the proportion of echinocandin-susceptible and echinocandin-resistant colonies in each of the 9 bottles, the following volumes were stroked onto Sabouraud plates in triplicate, depending on the spiked inoculum: 10 μl (a 1:10 dilution of the 1×10^3- to 5×10^3-CFU/ml inoculum was prepared to obtain single colonies), 10 μl (1×10^2 to 5×10^2 CFU/ml), and 100 μl (10 to 50 CFU/ml). The plates were then incubated at 35°C

for 48 h. We performed the EUCAST EDef 7.3.1 antifungal susceptibility test on single colonies (up to 10 colonies per bottle) for determination of susceptibility to micafungin and anidulafungin (21).

Five to 6 drops of the broth medium were stroked onto RPMI 1640 agar plates, and after placing the Etest strips for anidulafungin and micafungin, the plates were incubated for 24 h (ET_{DIR}). Isolates were classified as echinocandin resistant per the ET_{DIR} MICs using the same clinical breakpoints of the EUCAST microdilution method (30). In the absence of growth at 24 h, the anidulafungin-containing plates were incubated for 48 h. Isolates growing on anidulafungin-containing agar plates were considered echinocandin resistant, as reported elsewhere (16).

Statistical analysis. We calculated the total number of pooled resistant colonies from the bottles spiked with a given inoculum and compared the proportions of resistant colonies found in the three groups of bottles spiked with the different inocula (10^3, 10^2, and 10 CFU/ml). The comparison of proportions was done using a standard binomial method (95% confidence interval) (Epidat [version 3.1] software; Servicio de Información sobre Saúde Pública de la Dirección Xeral de Saúde Pública de la Consellería de Sanidade, Xunta de Galicia, Spain).

Ethical considerations. This study was approved by the Ethics Committee of the Hospital Gregorio Marañón (CEIC-A1; study no. 208/16).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02004-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We have no conflicts of interest to declare.

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7. DISCUSIÓN

El estudio de la infección fúngica invasora ha sido abordado fundamentalmente desde el punto de vista clínico debido a la alta morbi-mortalidad de los pacientes y al elevado coste económico para las instituciones. En los últimos años, se ha observado un cambio en la epidemiología de las especies de *Candida* causantes de infección, siendo *C. glabrata* una de las que más se han afectado. Esto es debido a que su incidencia ha aumentado considerablemente, debido al incremento en el número de pacientes propensos a adquirir infección.

Un factor determinante que ha centrado la atención en el estudio de *C. glabrata* es el aumento notable de la tasa de resistencia a las candinas, antifúngicos de primera línea en el tratamiento de estas infecciones. Esto es especialmente importante en una especie con baja susceptibilidad a los azoles. En la actualidad, la resistencia de *C. glabrata* a las candinas en España es baja, a diferencia de lo que ocurre en algunos hospitales de Estados Unidos de América y del norte de Europa. Esto conlleva a una continua vigilancia de la aparición de la resistencia, y a un estudio profundo del patógeno. Una de las características principales de *C. glabrata* en comparación con otras especies de *Candida*, es que ha demostrado la capacidad de adquirir resistencia de manera rápida a múltiples antifúngicos (Bartizal et al., 1997; Balashov et al., 2006; Locke et al., 2016; Shields et al., 2018). Sin embargo, a día de hoy, son pocos los pacientes infectados por aislados resistentes a las candinas. Del mismo modo, otras características propias de las cepas como su patogenicidad, la velocidad de crecimiento, la capacidad de formación de biopelículas, y su letalidad en modelos animales han recibido poca atención.

La aparición de resistencia en *C. glabrata* a las candinas parece estar relacionada con su predisposición a adquirir fácilmente mutaciones en los genes *FKS* en respuesta a la presión antifúngica, probablemente debido por su naturaleza haploide (Arendrup y Perlin, 2014; Perlin, 2014; Shields et al., 2014). La presión de estos fármacos sobre este patógeno puede darse tanto en su uso como profilaxis, como en el seno de tratamiento cuando este se prolonga en el tiempo (Thompson et al., 2008; Lewis et al., 2013; Alexander et al., 2013). Sin embargo, existen casos en los que se describe la aparición de resistencia a los pocos días del inicio del tratamiento (Lewis et al., 2013; Sasso et al., 2017), lo que sugiere que *C. glabrata* puede adquirir resistencia secundaria de forma rápida y sin elevada presión de antifúngico.

Como ha quedado demostrado en esta tesis, *C. glabrata* es capaz de desarrollar resistencia *in vitro* a las equinocandinas tras exposición a micafungina y/o anidulafungina (Bordallo-Cardona et al., 2017; Bordallo-Cardona et al., 2018b; Bordallo-Cardona et al., 2018d). La adquisición de resistencia pudo observarse en diferentes condiciones experimentales con exposiciones iniciales en placas que contenían bajas concentraciones de micafungina y cuando se expusieron los aislados posteriormente en pases sucesivos, tanto en placas con la misma baja concentración o con concentraciones progresivamente mayores. Se observó que los aislados mantenían su capacidad de crecer en esas circunstancias desarrollando mutaciones en los genes *FKS* y además, mostrando mayores

CMIs a micafungina y anidulafungina. La exposición, por tanto, a concentraciones bajas de equinocandinas supone un ambiente propicio para el desarrollo de resistencia, que a la larga termina seleccionando a los mutantes subrepresentados a costa de la población salvaje. En este sentido, uno de los aislados estudiados ilustró muy bien este fenómeno de coexistencia de poblaciones, ya que se detectaron dos mutaciones en el gen *FKS2* (S663P y D666Y) cuando el aislado se cultivó en la placa que contenía una baja concentración de micafungina (0,062 mg/L). Sin embargo, se observó un enriquecimiento en la mutación S663P a concentraciones más altas de micafungina (Bordallo-Cardona et al., 2017). Debido posiblemente a las mutaciones que aparecen en los "Hot Spot" de los genes *FKS*, que condicionan el fenotipo de resistencia a candinas (Arendrup y Perlin, 2014), y el cambio encontrado en el aminoácido S663 en el gen *FKS2* confiere una resistencia elevada, mientras que el cambio en D666 confiere una resistencia débil. Por otra parte, aunque la deleción F658 no se encuentra dentro del "Hot Spot", su presencia se ha asociado con CMIs elevadas (Alexander et al., 2013; Pham et al., 2014b). Aun así, se observó que una de las cepas con esta deleción adquirió resistencia a anidulafungina a bajas concentraciones durante la exposición y posteriormente a micafungina cuando se expuso a la concentración más alta del fármaco (Bordallo-Cardona et al., 2017). Otro fenómeno que se observó es que, algunas de las colonias moderadamente resistentes (1 o 2 diluciones por encima del punto de corte) a las equinocandinas obtenidas *in vitro* no albergaron mutaciones en los genes *FKS*. Diversos autores han corroborado estas observaciones en aislados de *C. glabrata*, así como fenotipos con CMIs altas a las candinas en cepas *FKS* salvajes, lo que justifica futuros estudios sobre esta cuestión (Pham et al., 2014b; Shields et al., 2018). Curiosamente en nuestros trabajos, la exposición de las mismas cepas a diferentes concentraciones del mismo antifúngico condujo a la generación de diferentes mutaciones en el gen *FKS2*. El impacto clínico de la CMI ha sido estudiado como un predictor de pronóstico/respuesta ya que, pacientes infectados con aislados con una CMI baja a micafungina y elevada a anidulafungina pueden responder al tratamiento con micafungina de las misma forma que un aislado de tipo salvaje (Arendrup et al., 2011c; Arendrup et al., 2012). Sin embargo esta cuestión debe ser evaluada en mayor profundidad en el futuro.

La capacidad de *C. glabrata* de adquirir resistencia secundaria *in vitro* podría explicar la compartimentalización de la resistencia, es decir, la presencia de aislados resistentes en determinadas zonas del organismo, donde se alcanzan concentraciones subterapéuticas de candinas. Estas observaciones pueden tener impacto clínico puesto que micafungina penetra débilmente en el líquido peritoneal, alcanzando un ratio líquido/plasma de 0,3 ($AUC_{0\text{-}24h}$) (Grau et al., 2015). De hecho, es en la cavidad abdominal donde se ha demostrado la promoción de mutantes que posteriormente provocan infección invasiva, en muchas casos tras la utilización de candinas (Shields et al., 2014). El mismo efecto de bajas concentraciones de candinas encontradas en mucosas y la promoción de mutantes descrita en ese órgano, podría ser debido a lo anterior mencionado (Jensen et al., 2015). Esto sugiere

que no alcanzar una exposición mínima a estos antifúngicos puede seleccionar mutantes que potencialmente podrían dar lugar a infecciones invasivas.

La exposición progresiva *in vitro* a concentraciones crecientes de micafungina, permitió a todos los aislados estudiados crecer hasta la máxima concentración de micafungina en placa estudiada (2 mg/L) (Bordallo-Cardona et al., 2017). Sin embargo, la exposición *in vitro* directa a esa misma concentración no permitió el crecimiento de las cepas. Esta observación es coherente con lo anteriormente observado, y probablemente se debe al hecho de que no hay tiempo para que se produzca una selección y enriquecimiento de mutantes, por lo que la población expuesta es exclusivamente salvaje y no soporta esas altas concentraciones de antifúngico (Bordallo-Cardona et al., 2018d). Se han propuesto parámetros (la frecuencia de mutación, la concentración preventiva de mutantes [MPC] y la ventana de selección de mutantes [MSW]) en antibacterianos que permitirían optimizar la respuesta al tratamiento y prevenir la aparición de aislados resistentes (Dong et al., 1999; Dong et al., 2000; Blondeau et al., 2001; Cantón y Morosini, 2011). Estos parámetros han recibido poca atención en el estudio de la infección por *C. glabrata*; sin embargo, en este sentido, las observaciones de esta tesis parecen sugerir una concentración mínima de candinas que sería deseable alcanzar para encontrarse en una "zona de seguridad". Según los resultados obtenidos, concentraciones de micafungina y anidulafungina ≥2 mg/L podrían prevenir la aparición de mutantes resistentes en *C. glabrata* (parámetro MPC) y concentraciones entre la CMI y la MPC permiten la aparición de mutantes (parámetro MSW) ya que, la exposición progresiva a concentraciones incluidas en la MSW (0,031 mg/L) dio lugar a la generación de mutantes (Bordallo-Cardona et al., 2018b; Bordallo-Cardona et al., 2018d).

Idealmente, la concentración de un fármaco en el lugar de la infección debe estar por encima de la MPC para minimizar la selección de mutantes. Sin embargo, esto sólo va a ser posible si la farmacocinética del fármaco lo permite, ya que no se pueden aumentar las dosis de fármaco ilimitadamente por la potencial aparición de toxicidad. Una posible solución es combinar fármacos para disminuir así la MPC, sin necesariamente administrar dosis superiores, pero en este momento la combinación de antifúngicos sólo se recomienda en situaciones concretas (Cornely et al., 2012; Pappas et al., 2016) y probablemente esté justificada en escenarios con una alta tasa de resistencia a las candinas. Como ejemplo de la potencial traslación de estos parámetros a la práctica clínica, las concentraciones séricas de micafungina, anidulafungina y caspofungina son 4,95 mg/L, 3,5 mg/L y 7,64 mg/L, respectivamente (Nicasio et al., 2009; Chen et al., 2011), mientras que la concentración en el peritoneo es <2 mg/L (Grau et al., 2015). Esto está línea con la baja tasa de resistencia encontrada en cepas aisladas de sangre frente a la mayor probabilidad de encontrarlas en la cavidad abdominal (Shields et al., 2014).

Sigue siendo una incógnita por qué algunas cepas de *C. glabrata* adquieren mutaciones de resistencia en los genes *FKS*. Diversos autores han propuesto que la

adquisición de resistencia a múltiples antifúngicos en diferentes especies de hongos, incluida *C. glabrata*, puede ser debida a defectos en la reparación del ADN (Legrand et al., 2007; Healey et al., 2016; Boyce et al., 2017). Un estudio reciente, identificó un fenotipo hipermutador en *C. glabrata* causado por un defecto en los genes de reparación de la replicación del ADN, exactamente en el gen *MSH2,* capaz de adquirir resistencia a múltiples antifúngicos (Healey et al., 2016), proponiendo la detección de mutaciones puntuales en el gen *MSH2* como un marcador potencial de desarrollo de resistencia (Healey et al., 2016). Sin embargo, estudios posteriores y los presentados en esta tesis, no parecen avalar esta teoría.

Se pretendió estudiar la correlación entre la presencia de mutaciones en el gen *MSH2* con (i) adquisición de resistencia *in vitro* e *in vivo*, (ii) el genotipo de los aislados y (iii) pronóstico de los pacientes y el uso previo de antifúngicos (Bordallo-Cardona et al., 2018a). La tasa de aislados que albergaron mutaciones en el gen *MSH2* (44,4%) encontrada en esta tesis fue similar a la informada en otros estudios (Healey et al., 2016; Singh et al., 2018), pero inferior al 77,2% observada por Hou y colaboradores (Hou et al., 2018). Ciertas mutaciones, como V239L y P208S/N890I, se han asociado con la adquisición de resistencia (Healey et al., 2016; Singh et al., 2018), a diferencia de otras menos comunes como E7K, E456D o E459K (Healey et al., 2016; Dellière et al., 2016; Singh et al., 2018). En esta tesis se observó lo siguiente: primero, los aislados de *C. glabrata* productores de primeros episodios y sensibles a las equinocandinas así como, sus respectivos aislados resistentes generados *in vitro,* tenían la misma secuencia del gen *MSH2*. Segundo, los aislados sensibles a equinocandinas con mutaciones en el gen *MSH2* o con una secuencia de tipo salvaje pudieron cambiar a un fenotipo resistente después de la exposición *in vitro* a las equinocandinas. En tercer lugar, el aislado incidente sensible a las equinocandinas del paciente con endocarditis que albergaba mutaciones en el gen *MSH2* fue capaz de producir *in vivo* aislados sensibles y resistentes a las equinocandinas. Finalmente, no se observó que las alteraciones en el gen *MSH2* influyeran en la resistencia al fluconazol. Además, no se encontró ninguna correlación entre la presencia de mutaciones en el gen *MSH2* y la adquisición de resistencia antifúngica, ya sea *in vitro* o *in vivo*. Este hallazgo está en línea con estudios previos, en los que también se destacó esta falta de correlación (Dellière et al., 2016; Singh et al., 2018; Hou et al., 2018).

Estudios anteriores, han relacionado diversos genotipos específicos de *C. glabrata* con mutaciones del gen *MSH2* (Dellière et al., 2016; Hou et al., 2018). Dellière y colaboradores, encontraron una correlación entre algunos genotipos definidos mediante microsatélites y mutaciones en el gen *MSH2* (Dellière et al., 2016); sin embargo, las mutaciones no fueron genotípico-específicas. Hou y colaboradores, observaron una correlación entre el ST7, ST10 y la presencia de ciertas mutaciones en el gen *MSH2* (V239L±K583N y P208S/N890I, respectivamente) (Hou et al., 2018); sin embargo, los autores no revelan si las mismas mutaciones también se encontraron en otros STs. Finalmente, los resultados descritos por Deshpande y colaboradores están en línea con los observados por

Hou exceptuando que, la mutación V239L, aparte de encontrarse en el ST7, también se encontró en el ST2, mientras que el genotipo ST3 estaba formado por aislados de tipo salvaje (Deshpande et al., 2018). Al considerar todos estos estudios, incluidos los presentados en esta tesis, parece haber una cierta relación entre el genotipo y la secuencia del gen *MSH2*; por ejemplo, el ST7 y el ST10 albergan mutaciones específicas mientras que, la mutación V239L, está presente en ST2, ST3, ST7, ST8 y ST34. Estos hallazgos sugieren que las mutaciones en el gen *MSH2* pueden ser posibles polimorfismos constitutivos, en lugar de mutaciones que podrían indicar el desarrollo de resistencia, como apoya un estudio reciente que utiliza datos de secuenciación de genoma completo en *C. glabrata* (Carreté et al., 2018). Además, la mayoría de las secuencias del gen *MSH2* fueron casi idénticas en los aislados que pertenecen a un genotipo.

Esta tesis no solo detalla la caracterización microbiológica de los aislados de *C. glabrata*, sino que también aporta una descripción clínica de los pacientes infectados. Los pacientes infectados por *C. glabrata* con mutaciones en el gen *MSH2* o de tipo salvaje presentaron características demográficas y clínicas similares. Además, no se encontraron diferencias entre los dos grupos en lo referente a la utilización previa de antifúngicos, como se ha observado en otro estudio previo (Dellière et al., 2016). Sin embargo se detectó un número mayor de casos graves (puntuación de Pitt alta, mayor incidencia de shock séptico y de mortalidad a los 30 días del diagnóstico de la candidemia en este grupo) en el grupo de pacientes infectados por cepas con el gen *MHS2* de tipo salvaje. Los pacientes con peor pronóstico estaban infectados con los genotipos ST3 y ST7, de la misma manera que otros autores han descrito que estos genotipos pueden causar un peor pronóstico (Byun et al., 2018).

La aparente contradicción entre la escasez de cepas clínicas invasoras resistentes a candinas y la facilidad con la que se pueden promover resistencia *in vitro*, no tiene una explicación clara. Una posibilidad es que las cepas resistentes muestren diferencias frente a las cepas salvajes en cuanto al "fitness" y/o virulencia (Perlin, 2011; Arendrup y Perlin, 2014; Perlin, 2015b). En esta tesis se ha evaluado la virulencia/patogenicidad como supervivencia en el modelo animal de *Galleria mellonella* y la formación de biopelículas y el "fitness" medido mediante parámetros cinéticos en cepas de *C. glabrata* tanto resistentes a las equinocandinas como salvajes.

La probabilidad de que las cepas mutantes tengan menor capacidad para subsistir en el paciente por su potencial coste biológico, podría explicar su baja probabilidad de ser detectadas. El modelo de *G. mellonella* comporta la inoculación de las cepas directamente dentro del organismo de la larva, por lo que con este modelo no se está evaluando la capacidad de las cepas de acceder a los tejidos profundos sino la de producir daño una vez allí. No se encontraron resultados concluyentes en relación con la mediana de supervivencia de las larvas infectadas por cepas de *C. glabrata* mutantes frente a las salvajes. Estos resultados están en línea con otros estudios anteriores (Lepak et al., 2012; Borghi et al.,

2014a). Además, otro estudio previo han demostrado que los aislados mutantes mantienen intacta la capacidad de invasión en los pacientes (Wiederhold, 2016). Queda pendiente estudiar si se encontrarían diferencias a la hora de atravesar barreras para acceder a órganos profundos por sí mismas.

Otro modelo que posibilita el estudio de la patogenicidad es el análisis de la capacidad de las levaduras para formar biopelículas. En esta tesis tampoco se encontraron diferencias significativas entre la producción de biopelículas por parte de los aislados mutantes de *C. glabrata* y las de sus respectivas cepas salvajes. *C. glabrata* se caracteriza por formar biopelículas con baja biomasa y alta actividad metabólica (Marcos-Zambrano et al., 2014a) mientras que *C. albicans* se caracteriza por su mayor capacidad de formar biopelículas con elevada biomasa. Por lo tanto, la falta de diferencias entre los grupos (salvajes y resistentes) no es sorprendente, debido a la menor mortalidad que en general presentan los pacientes infectados por *C. glabrata*, lo que potencialmente sea explicable por su baja capacidad de formar biopelículas, de la misma forma que los pacientes infectados por *C. albicans* presentan un peor pronóstico (Rajendran et al., 2015b).

Por último, se estudiaron los parámetros cinéticos de crecimiento mediante curvas de densidad óptica en diferentes especies de *Candida*, y se compararon aislados de *C. glabrata* salvajes, resistentes a fluconazol y a equinocandinas (Bordallo-Cardona et al., 2018f). Es la primera vez que se desarrolla un estudio de estas características para mostrar diferencias en los parámetros cinéticos de crecimiento inter e intra especie. Este es un tema controvertido en la actualidad, ya que algunos autores no han demostrado un claro impacto en el "fitness" cuando compararon aislados de *C. albicans* y *C. glabrata* sensibles y resistentes a las candinas (Ben-Ami et al., 2011; Arendrup et al., 2012).

En el análisis de las cepas sensibles se demostró que *C. glabrata* creció vigorosamente, mostrando la curva cinética más pronunciada que el resto de las especies. Dado que el inóculo inicial es similar en todas las especies, las diferencias cinéticas podrían explicarse por el alto número de células de *C. glabrata* por unidad de volumen en los pocillos, posibilitado por el pequeño tamaño celular de esta especie y/o al tiempo corto de germinación. Las especies restantes mostraron una velocidad media de crecimiento más lenta en el siguiente orden: *C. tropicalis*, *C. krusei*, *C. parapsilosis* y *C. albicans*. En la comparación de los parámetros cinéticos entre cepas de *C. glabrata* sensibles y resistentes a fluconazol no se observaron diferencias cinéticas en el crecimiento. Sin embargo, sí se observaron con los aislados de *C. glabrata* resistentes a las equinocandinas, que mostraron una velocidad media de crecimiento más baja que los aislados sensibles, independiente del tipo de mutación en el gen *FKS*.

La alta velocidad de crecimiento de *C. glabrata* en las condiciones descritas contrasta con el tiempo de positividad de los hemocultivos, ya que es significativamente menor que con otras especies (Lai et al., 2012; Huang et al., 2013; Cobos-Trigueros et al., 2013;

Gokbolat et al., 2017). Con la excepción de *C. glabrata*, el resto de las especies mostraron el mismo orden en lo relativo al tiempo de positividad que la velocidad media de crecimiento. *C. tropicalis* y *C. krusei* son normalmente las especies con menor tiempo de positividad, seguidas de *C. parapsilosis* y *C. albicans* (con tiempos similares) (Lai et al., 2012; Huang et al., 2013). Una explicación es que las condiciones de incubación y crecimiento ensayadas en esta tesis no son las mismas que las de los hemocultivos (no solo por el inóculo sino también por la composición del medio y la disponibilidad de los nutrientes). Para comprobar esto, se estudiaron diferentes inóculos de aislados de las especies comentadas (1-5 $x10^4$, 1-5 $x10^3$, 1-5 $x10^2$, 1-5 x10 UFC/mL) y se observó que la magnitud de los parámetros cinéticos disminuyó: la fase de latencia fue mayor con el inóculo más bajo, mientras que las diferencias entre las especies no cambiaron (datos no mostrados).

Las condiciones experimentales utilizadas para estudiar la cinética de crecimiento (medio de cultivo, inóculo y temperatura de incubación) fueron exactamente las mismas que las recomendadas en el procedimiento de EUCAST e imitaron al pocillo control de crecimiento libre de fármaco a lo largo del tiempo. La determinación de la CMI, según EUCAST, se debe realizar tras 24 ± 2 horas de incubación si la densidad óptica del pocillo control es de ≥0,2. En estas condiciones se observó que, primero, la morfología de las curvas cinéticas a las 24 horas permitió detectar que *C. parapsilosis* se encontraba en la fase exponencial, mientras que las especies restantes se encontraban en una fase de desaceleración (fase pre-estacionaria) donde la replicación celular probablemente esté disminuyendo como consecuencia de la competencia (Alsuhaim H, 2013). En segundo lugar, la curva cinética de crecimiento entre las 22 y 26 horas mostró resultados similares en todas las especies, excepto para *C. krusei*, donde se observó que la curva evolucionaba desde la fase pre-estacional (22 horas) hasta la fase post-estacional (26 horas) donde horas después se podría intuir canibalismo. Dado que la determinación de la CMI se realiza al final de la fase exponencial, no se encontraron grandes diferencias en las densidades ópticas entre las 22 y las 26 horas de incubación, pero sí en cada una de las cepas de una misma especie. La definición de CMI de azoles y candinas frente a *Candida* no exige ausencia de crecimiento completo sino disminución en comparación con el pocillo control, por lo que diferencias en el crecimiento en este pocillo a lo largo del tiempo podría conllevar a variación en la CMI. Queda la duda de en qué medida la curva de crecimiento se ve afectada por la presencia de un antifúngico, condiciones que deben ser estudiadas en un futuro.

Dado que la detección de resistencia antifúngica es útil para corregir y optimizar el tratamiento antifúngico, la aceleración en la obtención de los resultados es de gran importancia en el manejo del paciente. Como se ha comentado en el método de EUCAST, la densidad óptica en el pocillo control libre de fármaco debe alcanzar un valor mínimo de 0,2 después de 24 ± 2 horas de incubación (Arendrup MC et al., 2017). Sin embargo, este umbral podría alcanzarse antes de las 24 horas y reduciendo así, consecuentemente, el tiempo de incubación. Muy pocos aislados alcanzaron este umbral antes de 8-10 horas de incubación y

la mayoría de ellos (80%) requirieron incubaciones entre 10 horas (*C. glabrata*) y 24 horas (*C. parapsilosis*). Por lo tanto, acelerar la obtención de los resultados de la susceptibilidad antifúngica puede ser factible solo en *C. glabrata*, *C. tropicalis* y *C. krusei*, aunque hay ciertas consideraciones a tener en cuenta. Primero, anticipar los resultados unas horas, en comparación con la lectura regular a las 24 horas, puede no ser relevante. Segundo, anticipar las lecturas llevaría a establecer la CMI en la fase exponencial del crecimiento fúngico y se desconoce el impacto de este fenómeno. Finalmente, estudios futuros deben probar que el crecimiento de la levadura en presencia de antifúngicos permite la interpretación de resultados con tiempos de incubación más cortos.

En línea con lo comentado, los resultados de la sensibilidad antifúngica deberían anticiparse en la medida que sea posible. Aunque se prefieren los métodos de referencia de microdilución, estos no están exentos de inconvenientes ya que se requieren el cultivo puro de las cepas y sus resultados se demoran 48-72 horas desde el diagnóstico de la candidemia, con el consiguiente retraso en el inicio del tratamiento antifúngico apropiado. Una potencial alternativa para anticipar los resultados de sensibilidad antifúngica es estudiar el papel de métodos de difusión en agar independientes del inóculo de la cepa de forma que se puedan aplicar directamente sobre muestra clínica. El grupo demostró previamente que el Etest® realizado directamente de hemocultivos positivos (ET_{DIR}) era equivalente al procedimiento CLSI M27-A3a para descartar falsa resistencia en levaduras, detectar resistencia a caspofungina en basidiomicetos, y resistencia a fluconazol en *Candida*-no *albicans* (Guinea et al., 2010). En un estudio posterior se demostró el papel del ET_{DIR} para detectar resistencia a fluconazol en *C. albicans*, correlacionándose tanto con el método CLSI M27-A3 como con el EUCAST EDef 7.3.1 (Escribano et al., 2017). En esta tesis se ha demostrado además, que este método es también fidedigno a la hora de detectar resistencia a equinocandinas en *Candida spp.* utilizando EUCAST EDef 7.3.1 como método de referencia (Bordallo-Cardona et al., 2018c).

Con el fin de mejorar el potencial del ET_{DIR} para la detección de aislados resistentes y mutantes, se calculó el acuerdo categórico utilizando la secuencia del gen *FKS* como método de referencia. Este acuerdo fue muy alto, lo que demuestra la capacidad del ET_{DIR} para discriminar, en la mayoría de los casos, los aislados con mutaciones en los genes *FKS* de los de tipo salvaje. Sin embargo, se observaron errores mayores en tres aislados de *C. glabrata* que mostraron CMIs de anidulafungina y micafungina cercanas al punto de corte de EUCAST, y muy altas tanto en el ET_{SD} como en el ET_{DIR}, si bien las secuencias de los genes *FKS1* y *FKS2* eran de tipo salvaje. Los puntos de corte usando el ET_{DIR} que permitieron separar los aislados de tipo salvaje o sensibles a las equinocandinas de los aislados con mutaciones en los genes *FKS* y/o resistentes fueron: anidulafungina ≥0,19 mg/L y/o micafungina ≥0,06 mg/L. La realización del ET_{DIR} demostró que se pueden acelerar los resultados de sensibilidad a las equinocandinas en las 24 horas siguientes a la detección de la candidemia, de manera fiable, rápida, sencilla y económica.

Además de estudiar la técnica del ET_{DIR}, se evaluaron placas de agar que contenían una alta concentración de anidulafungina (2 mg/L) como método rápido de cribado de resistencia; se eligió anidulafungina por ser este fármaco más sensible para detectar aislados resistentes que micafungina. La concentración era la MPC de *C. glabrata* para las dos equinocandinas estudiadas, por lo que si el aislado no crecía en esta concentración, la probabilidad de que la cepa fuera resistente sería baja. Este método ha demostrado ser útil para el cribado de cepas de *Aspergillus fumigatus* resistentes a los azoles (Arendrup et al., 2017). Se encontró, sin embargo que este método no fue útil para el cribado de resistencia a equinocandinas en *C. albicans*, *C. tropicalis* y *C. parapsilosis,* posiblemente debido al efecto paradójico (Rueda et al., 2014; Marcos-Zambrano et al., 2017a). Sin embargo, el mejor acuerdo se encontró en *C. glabrata* ya que, el 100% de los aislados sensibles a las candinas no fueron capaces de crecer y más del 90% de los aislados resistentes crecieron en las placas que contenían una alta concentración de anidulafungina.

Los dos procedimientos comentados (ET_{DIR} y las placas con anidulafungina) permitieron también la detección de mutantes cuando estos estaban infrarrepresentados (Bordallo-Cardona et al., 2018e). De hecho, se detectaron incluso en proporciones tan bajas que el método de referencia no fue capaz de detectarlos. Esto puede explicarse porque el inóculo de los aislados que se prepara de acuerdo al procedimiento EUCAST (1-5 $x10^5$ UFC/mL) no es suficiente para detectar bajas proporciones de mutantes por la tasa de mutaciones a estos fármacos, tasa que se estudió y calculó en esta tesis como 10^{-8} en presencia de micafungina o anidulafungina en *C. glabrata* (Bordallo-Cardona et al., 2018d). Estos experimentos se realizaron simulando la carga de levaduras en sangre de pacientes con candidemia, estimada en ≤1 UFC de levaduras por mL (rango entre 0,1 y >1,000) (Pfeiffer et al., 2011), por lo que suponiendo una venopunción de 10 mL de sangre inoculados en las botellas, el inóculo más bajo introducido en las botellas sería 10-50 CFU/mL, lo que simula una carga de *C. glabrata* de 1-5 CFU/mL circulando en sangre. Sin embargo, el tiempo de respuesta de las placas de agar que contienen anidulafungina fue peor que el del ET_{DIR} ya que se requirió de 48 horas de incubación.

Como conclusión final de esta tesis, a pesar de que en España la resistencia a las candinas en *C. glabrata* sigue manteniéndose baja, se ha demostrado que esta especie tiene potencial para adquirir fácil y rápidamente resistencia a estos antifúngicos. Además, se ha confirmado que el ET_{DIR} es una técnica rápida y fiable para su uso en los laboratorios de Microbiología para detectar resistencia a equinocandinas en *C. glabrata*, suponiendo una herramienta a tener en cuenta si finalmente aumentan las tasas de resistencia en cepas provenientes de muestras clínicas.

8. CONCLUSIONES

1. La exposición progresiva *in vitro* a concentraciones crecientes o a concentraciones bajas y constantes de micafungina, pueden promover fácilmente resistencia a las equinocandinas en aislados de *C. glabrata* causantes de candidemia.

2. Concentraciones *in vitro* de equinocandinas por debajo de 2 mg/L promueven el desarrollo de resistencia secundaria a las equinocandinas en aislados de *C. glabrata* productores de candidemia. En cambio, concentraciones superiores previenen su aparición.

3. No se observó un impacto significativo en la virulencia estudiada mediante larvas infectadas de *G. mellonella,* entre los aislados de *C. glabrata* sensibles a candinas y los aislados con mutaciones en el gen *FKS2*.

4. La presencia de mutaciones en el gen *MSH2* en aislados de *C. glabrata* que causan candidemia no se correlacionó con la adquisición de resistencia antifúngica *in vitro* o *in vivo*, con genotipos específicos, ni con el uso previo de antifúngicos en los pacientes.

5. Se encontraron diferencias significativas inter e intra especie en los parámetros cinéticos estudiados en los aislados clínicos de diferentes especies de *Candida*, siendo *C. glabrata* la especie más rápida en tasa de crecimiento. La resistencia a las equinocandinas en esta especie podría tener un impacto en la cinética de crecimiento.

6. La aceleración de los resultados de sensibilidad antifúngica mediante el método de EUCAST puede ser posible para ciertas especies, particularmente para *C. glabrata*, *C. tropicalis* y *C. krusei*.

7. El Etest® de equinocandinas realizado directamente de hemocultivos positivos es un procedimiento rápido, fiable, y sencillo para detectar resistencia a las equinocandinas en diferentes especies de *Candida* spp. incluso, cuando las cepas de *C. glabrata* resistentes están infrarrepresentadas en la población.

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10. ANEXO

Anexo: Comunicaciones de primer autor a Congresos Internacionales derivados de los resultados de esta tesis.

2016: 26th European Society of Clinical Microbiology and Infectious Diseases (ECCMID).

- *In vitro* exposure to micafungin promotes echinocandin resistance in clinical *Candida glabrata* Isolates.

2017: 27th ECCMID.

- *Candida glabrata* can acquire in-vitro resistance to echinocandins after exposure to low micafungin concentrations.
- Mutant prevention concentrations (MPCs) of micafungin and anidulafungin against *Candida glabrata* clinical isolates.

2018: 28th ECCMID.

- The presence of *MSH2* polymorphisms in *Candida glabrata* is not a predictor of antifungal resistance acquisition.
- Agreement between EUCAST procedure and the Etest direct on blood samples in *Candida* spp. clinical isolates.
- *Candida* spp. clinical isolates causing candidaemia show differences in the kinetic parameters.

26th ECCMID Amsterdam, Netherlands 9 – 12 April 2016

In vitro exposure to micafungin promotes echinocandin resistance in clinical *Candida glabrata* isolates

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Background

Emerging echinocandin resistance in *Candida glabrata* has recently been reported in some hospital institutions.

We aimed to study how *C. glabrata* can acquired *in vitro* resistance after prolonged exposure to increasing concentrations of micafungin.

Methods

5 *Candida glabrata* isolates from patients with candidemia.

Micafungin, anidulafungin, and caspofungin MICs were determined using EUCAST EDef 7.2 ($MIC_{initial}$).

A loopful of each grown isolate was suspended YPD broth overnight.

Adjusted inocula ($3\text{-}4x10^5$ cfu/mL) were stroked on plates containing micafungin (0.031 mg/L) incubated for 24 hours at 35°C. If yeast growth, a loopful of cultured isolates was propagated on a plate containing the next two-fold micafungin concentration up to 2 mg/L.

The suspensions were also used to study the MIC at each propagation step ($MIC_{subsequent}$) and on the plates containing 2 mg/L of micafungin (MIC_{final}).

Geometric mean of the $MIC_{initial}$s, $MIC_{subsequent}$s and MIC_{final}s of the three echinocandins were compared by the Wilcoxon test.

fks1 and *fks2* genes were sequenced from suspensions used for $MIC_{initial}$, $MIC_{subsequent}$s and MIC_{final} determination.

Stability of phenotypic and genotypic resistance:
- The phenotypically resistant isolates used to study the MIC_{final} were cultured on micafungin-free Sabouraud dextrose agar plates incubated for 24 h at 35 °C. Further propagations until 7th day were performed.
- *fks* sequencing and EUCAST antifungal susceptibility testing was performed at each of the 7 propagation steps.

Molecular typing of the isolates was performed using 17 microsatellite markers.

Results

All 5 isolates were echinocandin-susceptible (Table 1 and 2), their *fks1* and *fks2* sequences were wild-type (Table 2), and were genetically unrelated.

Both $MIC_{subsequent}$s and MIC_{final}s of the three echinocandins were significantly higher than $MIC_{initial}$s (Table 1) ($P<0.05$).

Overall, there was a trend to observe higher MICs when the concentration of micafungin in the plates increased.

Table 1. Susceptibilities of the *C. glabrata* isolates to echinocandins before and after the exposure to micafungin.

	Micafungin MIC (in mg/L)		Anidulafungin MIC (in mg/L)		Caspofungin MIC (in mg/L)	
	Range	Geometric mean	Range	Geometric mean	Range	Geometric mean
$MIC_{initial}$	0.015	0.015	0.062	0.062	0.125	0.125
Progressive exposure						
$MIC_{subsequent}$	0.015-2	0.18[a]	0.031-4	0.56[a]	0.125->8	2.70[a]
MIC_{final}	0.062-4	1.15[b]	1-4	2.30[b]	4->8	10.56[b]

Comparisons between the geometric mean [a]$MIC_{initial}/MIC_{subsequent}$ and [b]$MIC_{initial}/MIC_{final}$, for which significant differences were reached ($P<0.05$).

All isolates grown on plates containing micafungin at 0.062 mg/L or 0.125 mg/L concentrations became resistant to one or more echinocandins when studied by EUCAST (Table 2).

At the 0.125 mg/L concentration, four isolates had *fks2* mutations and were micafungin and anidulafungin resistant whereas the remaining isolate (CG3) was *fks* wild-type and only resistant to anidulafungin (Table 2).

Mutations in *fks2* gen were found in the 5 isolates when grown at micafungin concentrations of 2 mg/L. **S663** and **delF658** were the most common substitutions found.

CG1 isolate grown on plates with micafungin at 0.062 mg/L showed 2 mutations, one conferring weak resistance (**D666Y**) and the other conferring strong resistance (**S663P**); the latter was also found when the isolate was grown in the presence of higher micafungin concentrations. CG3 isolate showed *fks2* gen substitutions on plates with micafungin concentration of 0.25 mg/L.

Table 2. Antifungal activity of micafungin, caspofungin, and anidulafungin and the *fks* substitutions before and after the progressive micafungin exposure.

	MICs (in mg/L) obtained by EUCAST and *fks* sequence [a]											
Isolates	**CG1**		**CG2**		**CG3**		**CG4**		**CG5**			
	MICs (MYC/AND/CAS)	*fks2*	MICs (MYC/AND/CAS)	*fks2*	MICs (MYC/AND/CAS)	*fks2*	MICs (MYC/AND/CAS)	*fks2*	MICs (MYC/AND/CAS)	*fks2*		
$MIC_{initial}$	0.015/0.062/0.125	WT	0.015/0.062/0.125	WT	0.015/0.062/0.125	WT	0.015/0.062/0.125	WT	0.015/0.062/0.125	WT		
$MIC_{subsequent}$ [MYC concentration in plates]												
0.031 mg/L	0.015/0.031/0.125	WT	0.015/0.062/0.125	WT	0.015/0.062/0.125	WT	0.015/0.031/0.125	WT	0.015/0.031/0.125	WT		
0.062 mg/L	**0.5/2**/8	**S663P D666Y**	0.015/0.062/0.25	WT	0.015/**0.125**/0.125	WT	0.015/0.062/0.125	WT	0.015/0.062/0.125	WT		
0.125 mg/L	**1/2**/>8	**S663P**	**0.125/0.5**/2	**S663Y**	0.031/**0.25**/4	WT	2/2/>8	**del F658**	2/2/8	**del F658**		
0.25 mg/L	**1/2**/>8	**S663P**	**0.125/2**/1	**S663Y**	0.031/**0.5**/>8	**del F658**	2/2/>8	**del F658**	2/2/>8	**del F658**		
0.5 mg/L	2/2/>8	**S663P**	**0.25/2**/2	**S663Y**	0.031/**2**/>8	**del F658**	2/2/>8	**del F658**	2/2/>8	**del F658**		
1 mg/L	2/4/>8	**S663P**	**0.5/2**/8	**S663Y**	0.031/**2**/>8	**del F658**	2/2/>8	**del F658**	2/4/>8	**del F658**		
2 mg/L	4/4/>8	**S663P**	2/2/4	**S663Y**	**0.062/2**/8	**del F658**	2/2/>8	**del F658**	4/4/>8	**del F658**		
GM MIC*	0.74/1.34/14.25		0.12/0.55/1.22		0.03/0.37/2.97		0.49/0.67/4		0.55/0.82/3.62			

[a] GM MIC: Geometric mean of the MICs obtained for each antifungal agent. Numbers in bold indicate phenotypic resistance. Clinical breakpoints for caspofungin are lacking when using EUCAST.

The *fks* substitutions and the phenotypic resistance were stable and reproducible.
Genotyping showed that each isolate before and after micafungin exposure was isogenic.

Conclusions

- Our observations show the potential of *C. glabrata* to rapidly acquire resistance when exposed to low echinocandin concentrations, probably owing to its haploid nature.
- MICs of caspofungin and anidulafungin were higher than those of micafungin.
- The clinical impact of these *in vitro* observations is unknown and deserved further investigations.

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Candida glabrata can acquire *in vitro* resistance to echinocandins after exposure to low micafungin concentrations

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Background and Objectives

- Echinocandin resistance in *Candida glabrata* isolates has emerged in some geographic areas. Resistant isolates show elevated echinocandin MICs and harbour mutations in *fks* genes.
- We studied the potential of *C. glabrata* to acquire *in vitro* resistance to echinocandins after exposing the isolates to low micafungin concentrations and the fitness of *fks* mutant isolates was studied by measuring growth kinetics and virulence in an *in vivo Galleria mellonella* model.

Methods

Candida glabrata isolates (n=5), from patients with candidemia, genetically unrelated.
Micafungin and anidulafungin MICs were determined according to EUCAST EDef 7.2 ($MIC_{initial}$).

Exposure to micafungin-containing agar plates:

- Isolates suspensions were adjusted to 4 – 6 x 10^9 (mean of 4.7±0.98 x10^9) CFU/mL, stroked on 0.031 mg/L micafungin-containing plates (100 µL), and incubated at 35ºC for 24 h.
- If growth was observed, a new adjusted suspension was propagated on 0.031 mg/L micafungin-containing plates up to nine propagation steps. The MICs of the echinocandins and *fks1* and *fks2* mutations were studied at each propagation step ($MIC_{subsequent}$).
- Geometric means of $MIC_{initial}$ and MIC_{final} (last propagation step) of micafungin and anidulafungin were compared. Genotyping proved the absence of contaminations.

Study of fitness of the wild type and the resulting *C. glabrata* mutant isolates:

- The *in vitro* growth kinetics of wild type isolates and mutant isolates were compared. Differences among the kinetics parameters (average growth rate, and time to maximum rate) were studied.
- We compared the mortality caused by wild type isolates and by *fks* mutant isolates (obtained in the last propagation step) on *Galleria mellonella*.
  - Ten *G. mellonella* larvae per isolate were infected with 10 µL of inocula ranging 3-7x 10^6 CFU per larva.
  - Larvae were incubated at 37º C up to 7 days post-infection and the number of dead larvae was scored daily.
  - Survival curves were obtained by the Kaplan–Meier method and differences were evaluated by Log-Rank; a *P* value of <0.05 was considered to be statistically significant.

Results

All isolates were initially echinocandin-susceptible (GM of micafungin/anidulafungin = 0.015 mg/L and 0.017 mg/L, respectively) but they became phenotypically echinocandin resistant after 2-4 days of exposure to micafungin (GM 2.64 mg/L and 2 mg/L, respectively) (*P* < 0.05) (Table).

Mutations in the HS1 of the *fks2* gene were found in all resistant isolates. S663P was the most frequent substitution, followed by other substitutions located outside the HS1 (deletion at F658), and a substitution newly described (W715L) (Table).

Table: Susceptibility and fks2 HS1 substitutions on the first phenotypic resistant propagation step

Isolates	Propagation day when the isolate become phenotypically resistant	EUCAST MIC (mg/L) MYC/AND			*fks2* HS1 substitution
		$MIC_{initial}$	$MIC_{subsequent}$	MIC_{final}	
1	2nd	0.015/0.03	2/2	2/2	S663P
2	4th	0.015/0.015	2/2	2/2	W715L
3	3rd	0.015/0.015	1/2	4/4	S663P
4	3rd	0.015/0.015	2/1	2/1	delF658
5	2nd	0.015/0.015	2/1	4/2	S663P

The kinetics parameters of wild type and *fks* mutant isolates were similar.

However, we found differences (*P* < 0.0001) in the median survival days of larvae infected by *fks* mutant isolates (5 days) and by wild type isolates (3 days) (Figure).

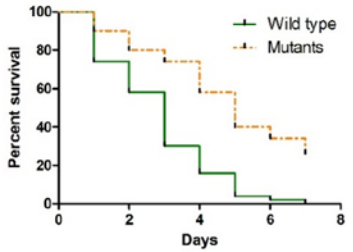

Figure. Survival curves of *G. mellonella* larvae infected by wild type isolates or infected by their corresponding mutant isolates.

Conclusions

- *C. glabrata* isolates can become resistant to echinocandins when exposed to low concentrations of micafungin. However, the resulting *fks* mutant isolates showed an attenuated virulence compared to the wild type isolates.
- These results suggest that the acquisition of resistance to echinocandins may hamper the pathogenicity of *C. glabrata*.

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Mutant prevention concentrations (MPCs) of micafungin and anidulafungin against *Candida glabrata* clinical isolates

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BACKGROUND and OBJECTIVE

- The mutant prevention concentration (MPC) is a parameter previously used to optimize antibacterial treatments minimizing the emergence of resistant isolates.
- We assessed the MPCs and the mutant selection window (MSW) of anidulafungin and micafungin against C. *glabrata* isolates with the corresponding mutation frequency.

METHODS

20 *C. glabrata* echinocandin-susceptible isolates

3-7x10^{9} CFU/mL

Streaked onto plates with micafungin/anidulafungin (0.015 to 2 mg/L)

Incubated up to 5 days

EUCAST MICs against the colonies growing onto plates containing 1 mg/L of micafungin or anidulafungin.

Sequence of *fks1* and *fks2* genes

- **MPC**: Lowest echinocandin concentration on the agar plates leading to complete inhibition of fungal growth.
- **MSW**: Range of concentrations between MIC and MPC.
- **Mutation frequency**: Ratio between the number of *fks* mutant colonies growing onto plates containing 1 mg/L of micafungin/anidulafungin and the number of cells streaked.

RESULTS

- The percentage of isolates growing on plates after 5 days of incubation is shown in the Table:

	Echinocandin concentrations (mg/L)							
	0.015	0.031	0.062	0.125	0.25	0.5	1	2
Anidulafungin (%)	100	100	100	100	95	70	50	0
Micafungin (%)	100	100	100	85	80	55	35	0

Table. Isolates growing on plates containing different concentration of micafungin and anidulafungin

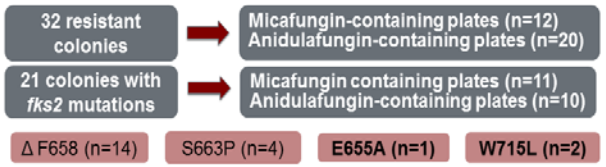
- Both echinocandins showed similar MPC ranges. The micafungin MSW was narrower than the anidulafungin MSW. Mutation frequency was lower in the presence of micafungin compared to anidulafungin (P=0.02) (Figure 1):

Figure 1. MPC and MSW range and mutation frequency

MYC	AND
MPC range (GM): 0.125-2 mg/L	MPC range (GM): 0.25-2 mg/L
MSW (GM): 0.015-0.73 mg/L	MSW (GM): 0.025-1.15 mg/L
M. frequency: 5.3-2.2 x10^{-8}	M. frequency: 3.9-1.7 x10^{-8}

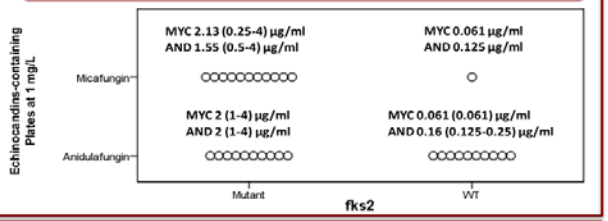
- A total of 12/20 isolates grew onto 1 mg/L micafungin/anidulafungin plates yielding 32 colonies phenotypically resistant as per EUCAST (Figure 2):

Figure 2. Colonies growing onto 1 mg/L candin-containing plates



- Mutations in bold have not been previously described
- Different mutations coexisted in four isolates
- Geometric mean MICs of anidulafungin and micafungin against mutant colonies and wild-type colonies were significantly different.
- Resistant colonies showing *fks* wild-type sequence were more frequently found on the anidulafungin-containing plate (Figure 3):

Figure 3: Colonies (n=32) growing on plates containing 1 mg/L of candins, and their corresponding fks2 sequence and range of MICs.



CONCLUSIONS

- Our study suggests that, overall, concentrations of anidulafungin and micafungin above ≥2 mg/L may prevent the emergence of *C. glabrata fks2* mutant isolates.
- In contrast, concentrations below 2 mg/L may be promote the development of secondary resistance to echinocandins.

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The presence of *MSH2* polymorphisms in *Candida glabrata* is not a predictor of antifungal resistance acquisition

Poster P0330

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Hospital General Universitario Gregorio Marañón

Introduction

- *C. glabrata* is prone to acquire echinocandin resistance and the presence of *MSH2* gene polymorphisms may have a role in the antifungal resistance.
- We here studied whether the *MSH2* gene sequence of a collection of candidemia *C. glabrata* isolates was associated to acquisition of antifungal resistance.

Methods

93 *Candida glabrata* isolates recovered from blood cultures of patients with candidemia **(93 episodes in 81 patients)** admitted to Gregorio Marañón Hospital (Madrid, Spain). The isolates included 20 ones previously exposed to anidulafungin and micafungin (Bordallo-Cardona MÁ and colleagues. Antimicrob Agents Chemother. 2018 23;62(3)), and an isolate causing endocarditis after a second episode of fungemia and showing a *FKS2* mutation (**S663P**).

All isolates were molecularly identified. Antifungal susceptibility to micafungin, anidulafungin, and fluconazole according to the EUCAST EDef 7.3.1 microdilution procedure was performed.

The *MSH2* gene was amplified and sequenced. All isolates were genotyped using a panel of 6 microsatellite markers. Singleton genotypes were defined as those found only once. Cluster was defined as the presence of ≥2 patients infected by an identical genotype.

Conclusion

- **The presence of *MSH2* mutations in candidemia *C. glabrata* isolates seems to be associated with specific genotypes rather than to the prediction of antifungal resistance acquisition.**

Results

- All isolates were echinocandin susceptible (geometric mean MICs of micafungin and anidulafungin were 0.0153 and 0.023 mg/L, respectively) and four of them (4.9%; Patients 11, 20, 44, 63) were fluconazole resistant (MICs ≥64 mg/L). Additionally, the isolate from a patient with endocarditis was micafungin and anidulafungin resistant (MIC=2 and 1 mg/L, respectively; genotype CG-6).
- A total of 11 different *MSH2* mutations were found in 44.4% (n=36/81) of the incident isolates [V239L (33.3%), P208S/N890 (25%), V239L/A942T (13.9%), E459K (8.3%) and, 2.7% of each of the following mutations (S653F, A313V, E456D, E7K, L588V, N890I and L810H) (Figure). *MSH2* sequencing of isolates causing second episodes yielded the same *MSH2* polymorphism found in the incident isolate. Two out of four fluconazole resistant isolates (Patient 11 and 20) harbored a mutation in the *MSH2* gene (E456D and P208S/N890I) (Figure and Table). Patient 15 was infected by a genotype causing a first episode of candidemia (echinocandin-susceptible isolate) and a second episode of endocarditis (echinocandin-resistant isolate); both isolates harbored two mutations in the *MSH2* gene (P208S/N890I).

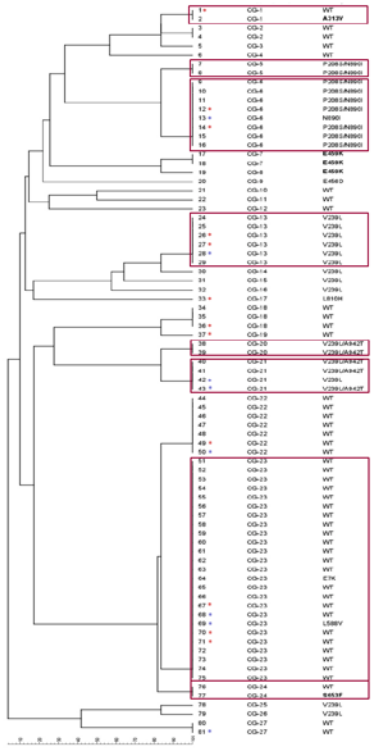
Table. EUCAST fluconazole MICs of 81 patients according to *MSH2* sequenced.

Fluconazole MIC EUCAST (mg/L)	WT	V239L	P208S/N890I	V239L/A942T	E459K	E456D	A313V	S653F	L810H	E7K	L588V	N890I
0.125-1												
2	2	1	1						1			
4	11	4	2	2	1			1				1
8	24	4	5	2	2		1					
16	5	1										
32	1	2		1						1	1	
64	1		1			1						
>64	1											
Total	45	12	9	5	3	1	1	1	1	1	1	1

Bold numbers, fluconazole resistant isolates.

- Genotyping of the 81 isolates, revealed the presence of 27 genotypes, 13 of which were clusters involving 82.7% of isolates (Figure). Isolates with *MSH2* mutations were found in 9 clusters, involving 78% (28/36) of mutant isolates. Most of the *MSH2* mutations were found in isolates involved in clusters and, although the bulk of isolates from each cluster showed the same *MSH2* polymorphism, some isolates showed an *MSH2* sequence that differed from the remaining isolates in the cluster.
- We found the V239L isolates in 7 genotypes (including 5 singletons), P208S/N890I in 2 genotypes, V239L/A942T in 2 genotypes, E459K in 2 genotypes (including 1 singleton), others 7 isolates with other mutations were in different genotypes, except E7K and L588V that were in the same genotype (Figure).
- *In vitro* acquisition of resistance to echinocandins was observed in 12/20 isolates. We found *MSH2* mutations in 5 induced echinocandin-resistant isolates and in 5 no induced echinocandin-resistant isolates. The remaining resistant isolates were *MSH2* wild type (Figure).

Figure. Genetic relationship among the 81 incident *C. glabrata* isolates studied.



***MSH2* mutations not previously described in bold.** Isolates exposed to echinocandins rendered either echinocandin-resistant isolates* or WT isolates*. Squares: clusters with any of the isolates showing *MSH2* mutations.

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Poster P0329

Agreement between EUCAST procedure and the Etest direct on blood samples in *Candida* spp. clinical isolates

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Introduction

- Rapid assessment of antifungal susceptibility of *Candida* isolates causing candidemia may help optimize antifungal treatment.
- We assessed the ability of the Etest and anidulafungin-containing agar plates performed directly on positive yeast blood cultures to rapidly study the echinocandin-susceptibility.

Methods

We prospectively collected 80 positive blood cultures with echinocandin wild type *Candida* spp. from patients with candidemia admitted to Gregorio Marañón Hospital (Madrid, Spain) and 35 echinocandin-resistant *Candida* spp. isolates were artificially spiked in Bactec bottles until they were flagged as positive [*C. albicans* (WT, n=20) (**F641S**, n=1); *C. tropicalis* (WT, n=20) (**F641L**, **S645F**, **R647G**, n= 3); *C. glabrata* (WT, n=20) (**Δ658**, n=14) (**E655A**, n=1) (**S663P**, n=7) (**S663Y**, n=1) (**D666N**, n=2) (**W715L**, n=3) (***FKS* WT echinocandin resistant,** n=3); *C. parapsilosis* (WT, n=20)].

A total of 2-4 drops of the broth medium (stored broth from the 80 isolates pre-incubated overnight at 37ºC and positive flagged bottles of the 35 echinocandin-resistant isolates) was stroked on RPMI 1640 agar plates on which Etest strips of micafungin and anidulafungin had been placed; 2-4 drops of the broth medium was also stroked on Sabouraud agar plates containing 2 mg/L of anidulafungin. Plates were incubated at 35ºC for 24 hours.

MICs obtained using EUCAST 7.3.1 were considered the gold standard and were compared with those obtained by ET_{DIR} and ET_{SD} to calculate essential agreement between the methods (percentage of isolates in which MIC differed by ±2 log dilutions over the reference method). All isolates were classified as resistant or susceptible according to the clinical breakpoints proposed by EUCAST for any of the three methods. Given the lack of clinical breakpoints for micafungin against *C. tropicalis*, we tentatively considered isolates showing an MIC above the ECOFF (> 0.06 mg/L). The procedures were in categorical agreement when the results were in the same susceptibility category based on two gold standards: EUCAST and *FKS* sequence (regardless of the MIC). The anidulafungin-containing plate screening procedure was in categorical agreement with EUCAST when resistant isolates or *FKS* mutants were able to grow on the plates and susceptible isolates or *FKS* wild types were unable to grow visibly on the plates. Errors were categorized as very major (false susceptibility) or major (false resistance).

Conclusions

- **ET_{DIR} for echinocandins a reliable and fast procedure when screening for the presence of echinocandin resistance in *Candida* spp. and can be easily implemented in the routine of the microbiology laboratory.**
- **Anidulafungin-containing plates cannot be recommended as screening tool unless *C. glabrata* is studied.**

Results

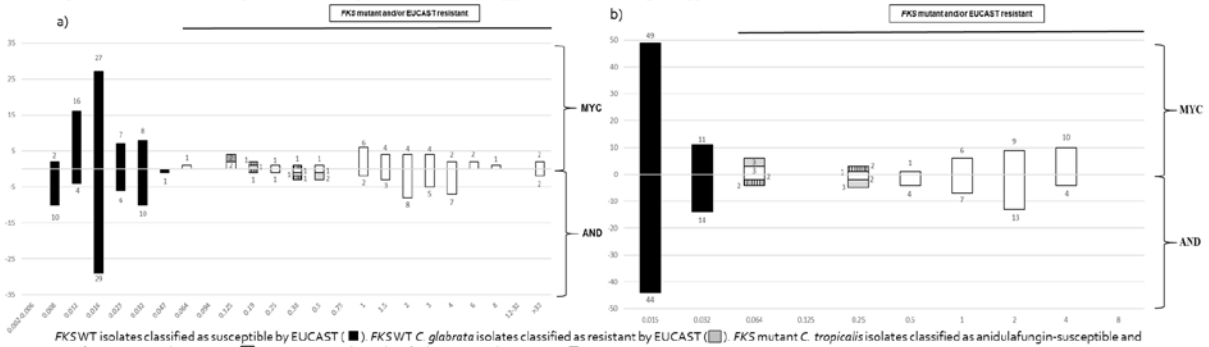
- Essential agreement between ET_{DIR}/ET_{SD} of micafungin and anidulafungin was 98.3% and 100%, respectively, with few exceptions (Table). Essential agreement between ET_{DIR}/EUCAST was >98% for micafungin and 100% for anidulafungin (Table).
- Categorical agreement between ET_{DIR}/ET_{SD} was 100% for all species and echinocandins. Categorical agreement between ET_{DIR}/EUCAST was 100% for micafungin and 98.3% for anidulafungin (Table). Misclassifications were found in two *C. tropicalis* isolates (8.7% of major errors) in which EUCAST indicated resistance to micafungin but susceptibility to anidulafungin, whereas ET_{DIR} and ET_{SD} indicated resistance to both drugs (Table); the *C. tropicalis* isolates harbored *FKS1* HS1 mutations (F641L and R647G).

Table: Essential agreement and categorical agreement between the methods for micafungin and anidulafungin.

Species	Essential agreement (% of isolates)				Categorical agreement (% of isolates)			
	ET_{SD} vs ET_{DIR}		EUCAST vs ET_{SD}/ET_{DIR}		EUCAST vs ET_{DIR}		*FKS* sequence vs ET_{DIR}/EUCAST	
	MYC	AND	MYC	AND	MYC	AND	MYC	AND
C. albicans	100	100	95.2/95.2	95.2/95.2	100	100	100	100
C. parapsilosis	100	95	100/100	90/95	100	100	100	100
C. tropicalis	100	100	100/100	95.6/95.6	100	91.3	100	100
C. glabrata	96.1	100	98/98.1	100/100	100	100	94.2	94.2
Overall	98.3	100	98.2/98.2	97.5/97.4	100	98.3	97.4	97.4

- Categorical agreement between ET_{DIR} and the *FKS* sequence was 97.4% for both echinocandins (Table); agreement was 100% for all species, with the exception of *C. glabrata* (94.2%), because of three isolates in which ET_{DIR} for both echinocandins (and EUCAST) indicated resistance but the *FKS1* and *FKS2* sequences were wild type (overall 2.6% of major errors). The figure shows the distributions of the micafungin and anidulafungin MICs obtained by ET_{DIR} and EUCAST (*C. parapsilosis* was excluded). An ET_{DIR} MIC of anidulafungin of ≥0.19 mg/L and/or an MIC of micafungin of ≥0.064 mg/L against *C. albicans*, *C. tropicalis*, and *C. glabrata* effectively separated the phenotypically resistant isolates/*FKS* mutants from the susceptible isolates/*FKS* wild types (100% categorical agreement with combined gold standards for both agents).

Figure. Distribution of micafungin and anidulafungin MICs obtained by ET_{DIR} (a) and EUCAST (b) against 95 isolates (*C. parapsilosis* isolates were excluded).



FKS WT isolates classified as susceptible by EUCAST (■). *FKS* WT *C. glabrata* isolates classified as resistant by EUCAST (▩). *FKS* mutant *C. tropicalis* isolates classified as anidulafungin-susceptible and micafungin resistant by EUCAST (▩). *FKS* mutant isolates classified as resistant by EUCAST (□).

- Overall, categorical agreement between EUCAST and growth on anidulafungin-containing plates was 62.6%. Major errors were found in all *C. albicans* and *C. tropicalis* echinocandin-susceptible isolates (EUCAST indicated susceptibility, but growth was visible on the plates [34.8%]). All *C. parapsilosis* were able to grow on the plates. The best agreement was observed for *C. glabrata* (94.2%), with 100% of susceptible isolates not growing on the plates and 90.3% of the resistant isolates growing on the plates. Very major errors (EUCAST indicated resistance but no visible growth on the plates, 2.6%) were found in the three echinocandin-resistant *FKS* wild-type *C. glabrata* isolates.

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Candida spp. clinical isolates causing candidaemia show differences in the kinetic parameters

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INTRODUCTION

- We studied the potential differences in the growth kinetics among clinically relevant *Candida* species isolates causing candidaemia. Furthermore, we assessed the potential impact of *FKS2* mutations in the kinetic parameters of *C. glabrata*.

METHODS

705 fluconazole and echinocandin-susceptible isolates from patients with candidemia admitted to Gregorio Marañón Hospital (Madrid, Spain) between 2007-2017.

+

31 echinocandin-resistant *C. glabrata* isolates obtained in previous studies were tested.

+

10 fluconazole-resistant *C. glabrata* isolates from patients with candidemia admitted to Gregorio Marañón Hospital and from the CANDIPOP project.

All 746 isolates were identified by amplification and sequencing of the ITS1-5.8S-ITS2 regions.

The same adjusted inocula prepared for EUCAST EDef 7.3.1 antifungal susceptibility to echinocandins and fluconazole was used for kinetic analysis.

Briefly, 100 µL of the suspension were added to double-concentrated RPMI 1640 medium in flat-bottomed microdilution trays and incubated with moderate shaking at 35ºC for 36 hours (performed in triplicate). The optical density in each well was measured every 15 minutes at 490 nm.

Differences among the kinetics parameters (average growth rate, time to maximum rate, maximum peak and, lag phase) were compared by Kruskal-Wallis and Mann–Whitney tests.

RESULTS

- Significant differences in kinetic parameters (average growth rate, time to maximum rate and, maximum peak) were found in *C. albicans* and *C. parapsilosis* ($P<0.01$) compared with the remaining susceptible species (Table). *C. glabrata* was the species showing the highest average growth rate and the highest maximum peak. However, *C. albicans* was the species showing the lowest average growth rate (Table).
- Average growth rate and time to maximum rate between susceptible *C. glabrata* isolates and fluconazole-resistant *C. glabrata* isolates were similar but higher than those found in echinocandin-resistant isolates ($P<0.001$). However, we did not found significant differences in the maximum peak between susceptible and resistant *C. glabrata* isolates (Table).
- Latent phase of *C. parapsilosis* isolates was significantly longer than that for the remaining species. *C. tropicalis* was the species with the shortest lag phase (Table). No differences were found between susceptible and resistant *C. glabrata* isolates.
- All species had an average growth rate that might mirror the time to positivity in blood cultures with the exception *C. glabrata* that paradoxically showed the fastest rate of growth.

Table. Kinetic parameters of susceptible and resistant species.

	Species	n	Average growth rate (s-1, mean)	Maximum peak (OD, mean)	Time to maximum rate (s, mean)	Lag Phase (h)
Susceptibles	*C. albicans*	351	3.87×10^{-6}	0.71	9.68×10^{4}	6.75
	C. parapsilosis	200	4.35×10^{-6}	0.66	9.52×10^{4}	8.75
	C. glabrata	83	7.50×10^{-6}	1.10	6.35×10^{4}	7
	C. tropicalis	54	6.90×10^{-6}	0.92	7.92×10^{4}	6
	C. krusei	17	6.08×10^{-6}	0.83	8.34×10^{4}	7.75
Echinocandin-resistant	*C. glabrata*	31	4.59×10^{-6}	1.05	1.04×10^{5}	7
Fluconazole-resistant	*C. glabrata*	10	7.98×10^{-6}	1.01	6.39×10^{4}	7.5

CONCLUSIONS

- Differences in the kinetics parameters among *Candida albicans* and *Candida parapsilosis* were found.
- The low average growth rate of *C. glabrata* echinocandin resistant isolates could have an impact on positivity time of blood culture.